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PHD

Flk-1 signalling during ES cell differentiation

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Flk-1 signalling during ES cell differentiation

Submitted by Lisa Angharad McRae

For the degree of Doctor of Philosophy

University of Bath

Department of Pharmacy and Pharmacology

September 2007

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Abstract

Flk-1 (Foetal liver kinase-1), a receptor tyrosine kinase, and its ligand VEGF (vascular endothelial growth factor) are essential for vasculogenesis and haemopoiesis in the early embryo. Flk-1 is expressed initially on the haemangioblast, the common precursor of haemopoietic and endothelial cells, and on subsequent committed endothelial lineages. Flk-1 expression is maintained on adult endothelial cells and mediates angiogenesis, making it an important pharmaceutical target in the pathology of many human diseases including cancer and rheumatoid arthritis. Though involvement of Flk-1 in disease has lead to its extensive study in adult humans little is known about the signals it mediates during development.

Using ES cells as a model of development, the aim of this investigation was to identify signals mediated through Flk-1 during early development and to characterise their relative importance in the formation of the haemopoietic, endothelial and cardiomyocyte lineages. Activation of the MAPK and PLC signalling pathways were demonstrated following VEGF treatment of Flk-1-expressing embryoid body-derived cells, though surprisingly activation of Flk-1 did not appear to mediate activation of the PI3K signalling pathway. Use of an embryoid body-based endothelial sprouting assay demonstrated a requirement for Flk-1 in both endothelial specification and angiogenic expansion. However, this activity was not mediated through either the MAPK or PI3K pathways. The finding that the PI3K pathway is not activated following VEGF stimulation nor required for early vasculogenesis/angiogenesis is surprising given its important role in both homeostatic and pathological angiogenesis in the adult. Previous work had suggested a role for Flk-1 in cardiac differentiation. Investigation of cardiomyocyte differentiation using Flk-1 null ES cells demonstrated a delay in formation of beating cardiomyocytes suggesting that Flk-1 may be involved in, but not required for cardiomyocyte specification. Finally, Flk-1-Tet-on ES cell lines were generated to facilitate investigation of the temporal importance of Flk-1 signalling during different developmental processes. Due to unforeseen difficulties in the maintenance of Flk-1 expression upon ES cell differentiation the full potential of this system was not realised in this study.

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Abbreviations

AcLDL	Acetylated low density lipids	
AGM	Aorta-Gonad-Mesonephros	
Bad	Bcl associated death promoter	
BL-CFC	Blast Colony Forming Cell	<i>In vitro</i> equivalent of the haemangioblast
BMP	Bone Morphogenic Protein	
BSA	Bovine Serum Albumen	
CD	Cluster of Differentiation	
cDNA	complementary DNA	
DAG	Diacylglycerol	
DMSO	Dimethylsulphoxide	
DNA	Deoxyribonucleic Acid	
Dox	Doxycycline	
E	Stage of embryonic development, days post coitum	d.p.c
EB	Embryoid Body	
EC	Embryonal Carcinoma	

ECL	Enhanced chemiluminescence	
EDTA	Ethylenediamine-tetraacetic acid disodium salt	
eNOS	Endothelial nitric oxide synthase	
Epo	Erythropoietin	
ERK1/2	Extracellular signal related protein kinase 1/2	
ES	Embryonic Stem (cells). Unless otherwise stated ES refers to murine embryonic stem cells	
FACS	Fluorescence Activated Cell Sorter	
FAK	Focal Adhesion Kinase	
FCS	Foetal Calf Serum	
FGF	Fibroblast Growth Factor	
Flk-1	Foetal Liver Kinase	KDR, VEGFR-2
Flk-1-Tet-on	Flk-1 ^{-/-} ES cell line stably expressing doxycycline inducible Flk-1 under the control of the pCAGGS-irtTA-VP16-puro tetracycline regulator and the pInshygro-tetTK-Flk-1-pA tet-response construct	
Flt-1	<i>fms</i> -like tyrosine kinase-1	VEGFR-1
Flt-4	<i>fms</i> -related tyrosine kinase-4	VEGFR-3

Gab1	Grb-2-associated binder-1
GATA	GATA binding protein
GFP	Green Fluorescent Protein
Grb2	Growth Factor Receptor bound protein-2
GSK-3	Glycogen Synthase Kinase-3
hES	Human embryonic stem (cells)
HIF	Hypoxia Inducible Factor
HLA	Human Leukocyte Antigen
HMVECs	Human microvascular endothelial cells
HRE	Hypoxia Response Element
HRP	Horse Radish Peroxidase
HSC	Haemopoietic Stem Cell
HUVECs	Human umbilical vein endothelial cells
ICM	Inner Cell Mass
Id	Inhibitor of differentiation
Ig	Immunoglobulin

ihh	Indian hedgehog	
IL	Interleukin	
irtTA	Reverse-tet-transactivator	
Jak	Janus kinase	
Kb	Kilobase Pairs	
KDa	KiloDaltons	
KDR	Kinase Domain Receptor	VEGFR2, Flk-1
LIF	Leukaemia Inhibitory Factor	DIA
Lmo2	Lim domain only 2	
MAPK	Mitogen Activated Protein Kinase	
MEFs	Murine Embryonic Fibroblasts	
MEK	Map-Erk-kinase	
mRNA	messenger RNA	
MTG	Monothioglycerol	
mTOR	mammalian target of rapamycin	
Nck	Non-catalytic region of tyrosine kinase adaptor protein	

P/Sp	Para-aortic-splanchnopleure	
PBS	Phosphate buffered Saline	
PCR	Polymerase Chain Reaction	
PDGF	Platelet Derived growth Factor	
PDK-1	3-phosphoinositide-dependent protein kinase-1	
Pecam-1	Platelet/endothelial cell adhesion molecule	CD31
PI3K	Phosphoinositide 3-kinase	
PIGF	Placental Growth Factor	
PILSAP	Puromycin Insensitive Leucine Specific Amino Peptidase	Arts1
PKB	Protein Kinase B	Akt
PKC	Protein Kinase C	
PLC	Phospholipase C	
PTEN	Phosphatase and tensin homologue	
r.p.m	rotations per minute	
RNA	Ribonucleic Acid	
RT-PCR	Reverse Transcription-PCR	

Runx	Runt-related transcription Factor	
S6	S6 ribosomal protein	
Scl	Stem Cell Protein	Tal1
SCNT	Somatic Cell Nuclear Transfer	
SDS-PAGE	Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis	
SH2	Src-homology domain	
Shb	src homology 2 domain-containing transforming protein B	
SHP-1 and 2	Src-homology 2 containing phosphatase-1 and 2	
Smads	Similar to Mothers Against Decapentaplegic	
Sox STAT3	SRY-box containing gene Signal Transducer and Activator of Transcription 3	
TBS	Tris Buffered Saline	
tet	Tetracycline	
TGF	Transforming Growth Factor	
Tie-1 and 2	Tyrosine kinase receptor-1 and 2	

TK	Thymidine kinase promoter	
TPO	Thrombopoietin	
Ve-Cadherin	Vascular endothelial Cadherin	
VEGF	Vascular Endothelial Growth Factor	
VEGFR	VEGF receptor	
VRAP	VEGF-Receptor Associated Protein	TSad, SH2 domain protein 2A

Chapter 1

Introduction

1.1 Stem cells

Stem cells were first brought to prominence by the pioneering work of Till and McCulloch which identified for the first time the Haemopoietic Stem Cell (HSC) (McCulloch and Till, 1964). Stem cells can be defined as unspecialised cells which have the capacity to self-renew (to produce daughter stem cells) and differentiate into more specialised cell types. Different types of stem cells exhibit different constraints in their abilities to differentiate. Embryonic stem (ES) cells, derived from the inner cell mass of the pre-implantation blastocyst are pluripotent meaning they can potentially differentiate into any cell type of the adult organism. Other pools of stem cells in the developing fetus and the adult are multipotent, meaning they have the potential to differentiate into multiple organ-specific cell types. For example, haemopoietic stem cells found in the adult bone marrow can differentiate into any blood cell but are largely considered restricted to the haemopoietic lineage. The ability to self-renew is unusual in the adult organism as most of its constituent cells are terminally differentiated and lack the ability to divide. Stem cells maintain and replace tissues that rapidly turn over such as the gut, skin and blood.

1.1.1 Therapeutic potential of stem cells

The ability of stem cells to differentiate into specialised cells has provoked much research into their potential therapeutic uses in providing a source of replacement cells or tissues. Conditions with the potential for stem cell treatment include Parkinson's disease, Alzheimer's disease, diabetes, cardiac ischaemia, liver diseases, spinal cord injuries and other degenerative diseases and injuries (Bobis et al., 2006; De Sousa et al., 2006; Keller, 2005; Pessina and Gribaldo, 2006). Figure 1-1 illustrates the stem cell hierarchy and potential therapeutic stem cell populations.

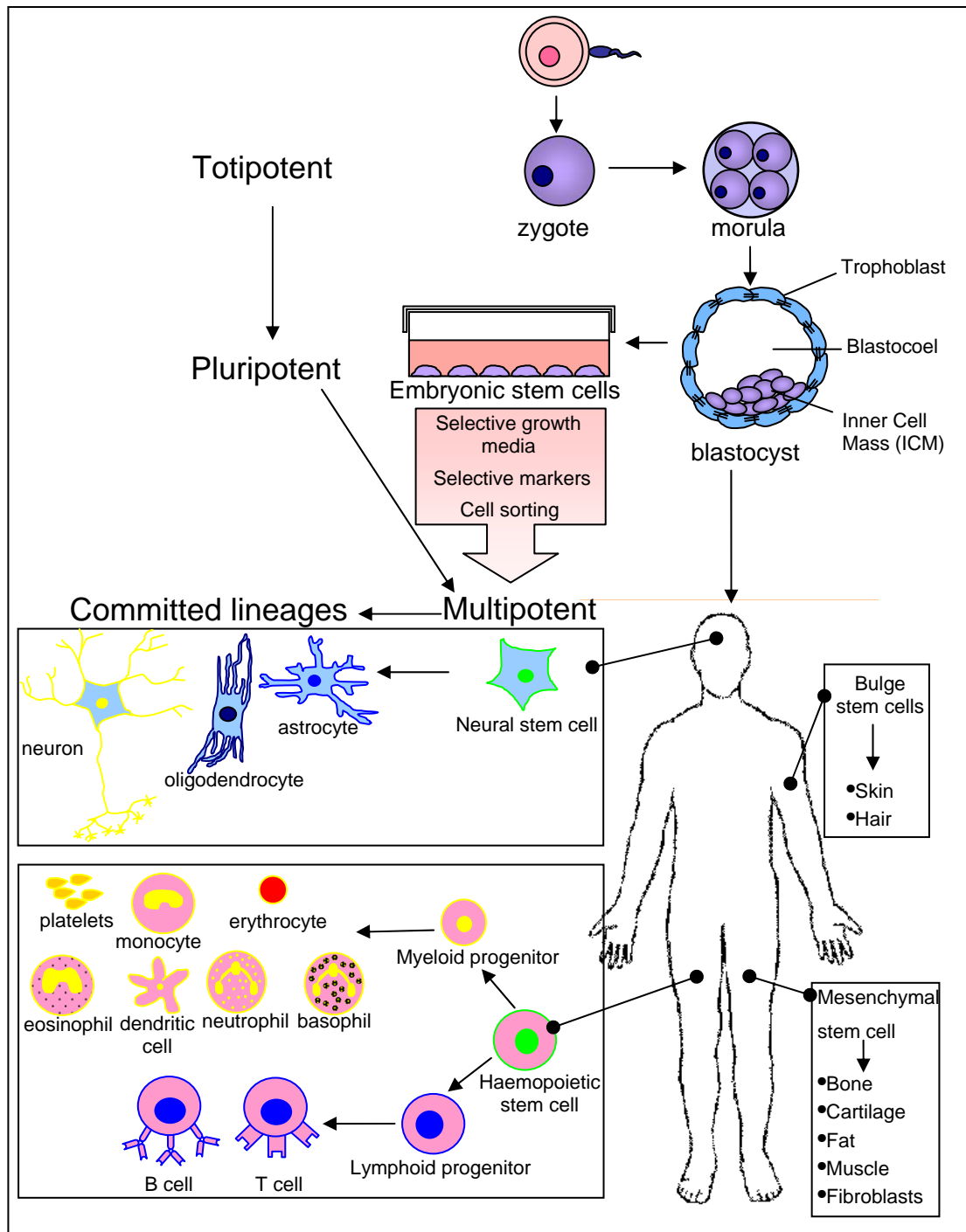


Figure 1-1 The stem cell hierarchy. Both the zygote and morula are totipotent meaning they can form any tissue of the adult organism and the extra-embryonic tissues. The inner cell mass (ICM) of the blastocyst forms the embryo proper and is the source of embryonic stem (ES) cells. Both the ICM and ES cells are pluripotent meaning they can form any tissue of the adult organism, but not extraembryonic tissues. Adult stem cells are multipotent as they have the ability to differentiate into more committed cells of a specific lineage. This figure illustrates some of the cells types which could be derived from both adult and embryonic stem cells. Diagram adapted from (Eckfeldt et al., 2005)

Treatment utilising some adult stem cells is already in place. Bone marrow is a source of haemopoietic stem cells used to treat blood disorders such as leukaemia (Amos and Gordon, 1995) and limbal stem cells from the eye can be used to replace a damaged cornea (Boulton and Albon, 2004). For most other cell and tissue types however this ultimate goal is still many years away. Adult stem cells are scarce, difficult to identify and isolate and don't generally grow well in culture making it difficult to expand them into useful numbers of cells. They are also generally thought to be lineage restricted, though some research into adult stem cells, including haemopoietic and mesenchymal (the stem cells of the bone, cartilage, muscle, fat and fibroblasts) stem cells has indicated that they may retain plasticity and given the correct microenvironmental cues could differentiate into other cell types. Currently the incidence of this is too low to be therapeutically beneficial and a great deal of controversy still surrounds the mechanism of these rare events (Oreffo et al., 2005; Wagers and Weissman, 2004).

Human embryonic stem cells (hES cells) were isolated in the late 1990s (Thomson et al., 1998) and like murine embryonic stem cells appear to have the capacity to differentiate into any cell type of the adult. However, as with adult stem cells, research into the maintenance of human ES cell self-renewal in large scale cultures and without feeder cells, along with the ability to derive pure populations of differentiated progeny, are largely unresolved and the field is still in its infancy. Furthermore, the use of human embryonic stem cells compared with adult stem cells holds greater ethical and regulatory concerns. Though the UK has a permissive policy regarding human embryonic stem cell research, countries such as Germany, Ireland and Norway have banned it and government funding is restricted in the USA (Check, 2005; Guenin, 2001; Munn et al., 2001).

An additional hurdle to overcome in the therapeutic use of any stem cells is the state in which to transplant them. Undifferentiated stem cells carry with them an inherent risk of cancer formation when injected *in vivo* (Roy et al., 2006). A test of pluripotency of embryonic stem cells involves their injection into a mouse leading to formation of a teratoma, a tumour containing cells and tissues from all three germ layers. For the replacement of many tissues, implantation of cells alone may not be enough for full reconstitution. More than one cell type may be required, plus it may

be necessary to seed and differentiate cells on a scaffold (tissue engineering) (Polak and Bishop, 2006). Lastly, as with any transplanted tissue, stem cell treatment would provoke an immune response. Only autologous transplants, were appropriate, or Somatic Cell Nuclear Transfer (SCNT in which a fibroblast cell from the patient is fused with an enucleated donor oocyte and patient matched embryonic stem cells are isolated from the resulting embryo) could fully get round this problem. SCNT has not yet been successfully carried out in humans and could be prohibitively expensive (Boyd et al., 2005; Priddle et al., 2006). Providing sufficient human ES cell lines to provide a tissue match for any potential patient would be a huge undertaking. Estimates comparing blood group and HLA types of cadaveric organ donors (as a surrogate for donor embryos) with the UK kidney transplant waiting list concluded that 150 hES cell lines would provide a full match for HLA –A (Human Leukocyte Antigen-A), HLA-B and HLA-DR for less than 20% of patients and that increasing the pool further provided little additional benefit (Taylor et al., 2005).

Though there is much promise for the therapeutic future of stem cells, the field is still in its infancy and much of the basic research has yet to be conducted. The fastest route to a role in therapeutics may be as models of different tissue types or disease states in drug discovery (McNeish, 2004).

1.1.2 Embryonic stem cells

Embryonic stem (ES) cells were first derived from the pre-implantation blastocyst of the mouse in 1981 (Evans and Kaufman, 1981; Martin, 1981) and have since also been derived from medaka (kill fish) (Wakamatsu et al., 1994), rabbit (Moreadith and Graves, 1992), chicken (Pain et al., 1996), pig (Wheeler M B, 1994), non-human primate (Thomson et al., 1995) and human embryos (Thomson et al., 1998). ES cells possess the potential to differentiate into any cell type of the organism, a property known as pluripotency. This potential has been demonstrated particularly well in mouse and chicken where embryonic stem cells cultured *in vitro* have, upon re-implantation contributed to the whole organism including the germ line (Nagy et al., 1993; Pain et al., 1996). Current and potential uses of embryonic stem cells are illustrated in Figure 1-2.

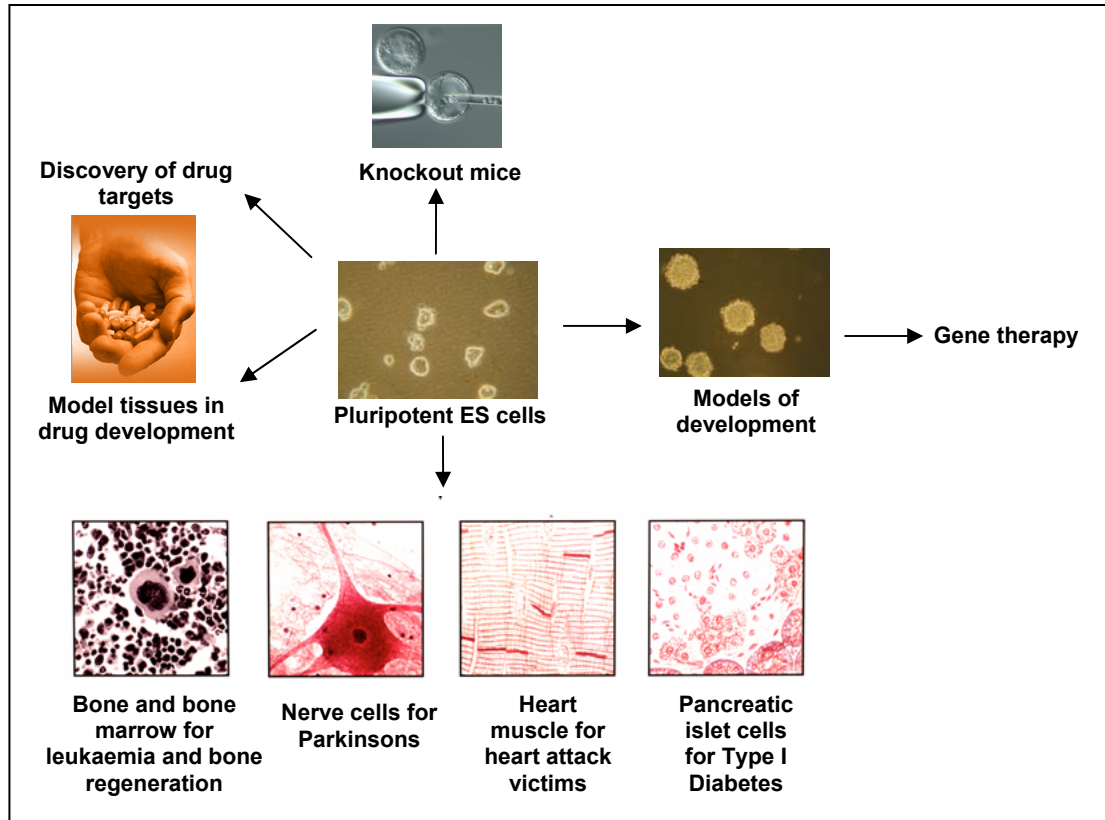


Figure 1-2 Current and potential uses of embryonic stem cells. Diagram adapted from ‘stem cell information’ available at <http://stemcells.nih.gov/info/media/defaultpage> accessed 15th February 2007.

1.1.2.1 A historical perspective – teratocarcinomas and EC cells

Prior to the derivation of embryonic stem cells other pluripotent embryonic cell lines had been studied. Embryonic carcinoma (EC) cell lines were isolated after it was observed that the engraftment of an early mouse embryo into an adult mouse led to the formation of a teratocarcinoma, a malignant multi-differentiated tumour containing a significant proportion of undifferentiated cells (Solter et al., 1970; Stevens, 1970). Teratocarcinoma formation only occurred upon implantation of pre-gastrulation embryos, containing the epiblast region, suggesting the transient epiblast population was predisposed for transformation into an embryonal carcinoma (Diwan and Stevens, 1976; Martin, 1980). The undifferentiated component of these embryo-derived teratocarcionomas behaved in the same manner as spontaneously arising teratocarcinomas. Cells could be propagated in culture and undergo differentiation *in vitro*, or form further teratocarcinomas producing cells of each of the germ layers (Kleinsmith and Pierce, 1964; Martin and Evans, 1975). In some cases (albeit at low efficiency due to aneuploidy in most embryonal carcinoma cell lines) these cells

could contribute to the embryo and produce chimeric offspring (Brinster, 1974). The finding that embryonal carcinoma cell lines were best maintained in co-culture in particular on mitotically-inactivated embryonic fibroblasts paved the way for the derivation of embryonic stem cells from the pre-implantation embryo (Martin et al., 1977).

1.1.2.2 Derivation of embryonic stem cells

Figure 1-1 illustrates the derivation of mouse embryonic stem cells. A number of different methods have been used to derive ES cells including the use of embryos undergoing delayed implantation (Evans and Kaufman, 1981), immunosurgical isolation of inner cell masses and derivation of ES cell lines in feeder-free conditions (Nichols et al., 1994; Ying et al., 2003). Most commonly ES cells are isolated on a feeder layer of mitotically inactivated Mouse Embryonic Fibroblasts (MEFs). Day 3.5 pre-implantation blastocysts are plated in DMEM supplemented with FCS and Leukaemia inhibitory factor (LIF) on MEFs. Over 2 days the embryos hatch from the zona pellucida and the trophoblast spreads out forming a monolayer of cells on which the inner cell mass can be seen. The inner cell mass is cultured for a further 2 to 4 days (before a core of endoderm forms around it) and is then either mechanically disaggregated or trypsinised and replated onto fresh MEFs. The disaggregated inner cell mass gives rise to ES cell colonies and other cell types. Over 7-10 days the ES cell colonies will be apparent, as other colonies differentiate and cease to proliferate, and the ES cells can then be expanded in culture (Abbondanzo et al., 1993; Nagy et al., 1993). Curiously, it has not been possible to derive ES cells from all mouse strains. Most widely-used embryonic stem cell lines, including R1 and E14, have been derived from the 129/Sv strain. ES cell lines from other inbred strains, including the C57BL/6J and C3H/He have been successfully derived, though generally with much lower efficiency (Abbondanzo et al., 1993; Gardner and Brook, 1997). There are many established protocols for the maintenance of ES cells in culture, both in the presence and absence of feeder cells. These will be discussed more fully in section 1.1.2.3 Maintenance of self-renewal.

1.1.2.3 Maintenance of self-renewal

The definition of self-renewal in stem cells is ‘the ability of a stem cell to give rise to a daughter cell with equivalent developmental potential’. This can be achieved by

either symmetric or asymmetric cell division. In the adult, asymmetric stem cell division enables maintenance of the stem cell pool, and simultaneous commitment of the second daughter cell to a more differentiated cell type required for tissue maintenance or repair. Symmetrical self-renewal, producing two identical daughter cells, is one of the defining features of *embryonic* stem cells. Understanding the mechanisms by which this is regulated is important if ES cells are to be derived and maintained in a defined medium in mass culture for both therapeutic and research purposes. Figure 1-3 gives an overview of the signals known to be involved in the regulation of murine ES cell self renewal which are described below.

Initially ES cells were grown on mouse fibroblast feeder layers to maintain them in an undifferentiated state. This is still common practise but advances in our understanding of the control of self-renewal have enabled the culture of mouse embryonic stem cells in medium containing a more defined serum replacement or in some cases in completely chemically defined medium (Wiles and Johansson, 1999; Ying et al., 2003). These advances have uncovered both extrinsic and intrinsic mechanisms important for the maintenance of self-renewal, demonstrated some of the similarities and differences in these mechanisms between mouse and human ES cells and revealed the complexity of a system still to be fully elucidated.

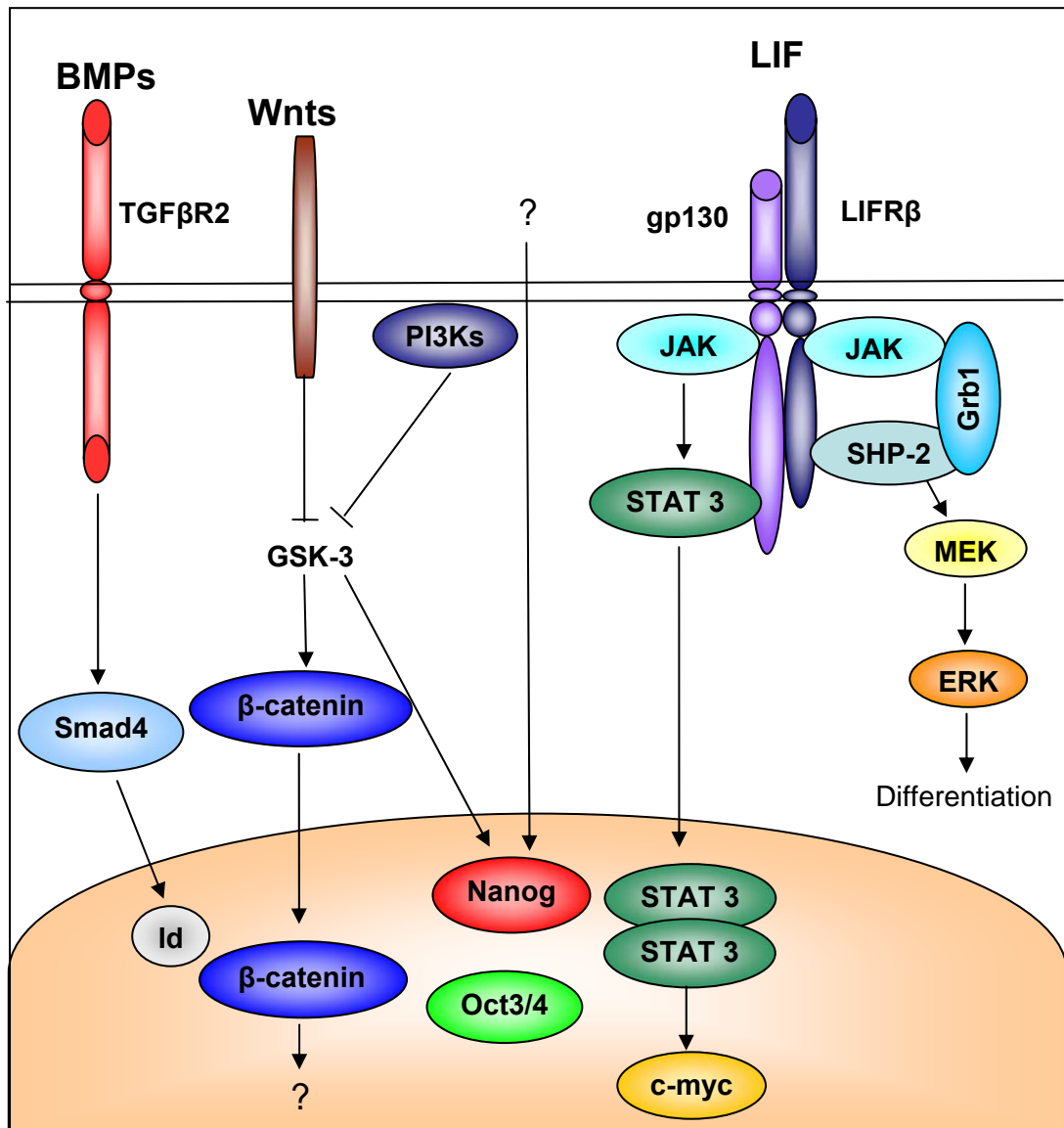


Figure 1-3 Signalling pathways regulating murine ES cell pluripotency. Diagram adapted from (Boiani and Scholer, 2005; Welham, 2007)

1.1.2.3.1 Signalling pathways with important roles in self-renewal

The first molecular signalling pathway demonstrated to be important for the maintenance of mES cell self-renewal was elucidated following the discovery that conditioned medium from Buffalo rat liver cell feeders was able to replace the feeders and sustain self-renewal. This led to the purification of differentiation inhibitory factor (DIA) (Smith et al., 1988) which was able to maintain self-renewal in the presence of foetal calf serum (FCS) without the need of a feeder cell layer. Simultaneously the previously cloned Leukaemia Inhibitory Factor (LIF) (Gearing et al., 1987) was also found to maintain the developmental potential of mES cells. DIA and LIF were subsequently identified as one and the same (Williams et al., 1988).

LIF was found to bind to a heterodimeric gp130/LIFR β receptor (Davis et al., 1993) causing phosphorylation and activation of associated Jak2 kinases (Janus Kinase 2; illustrated in Figure 1-3) (Narazaki et al., 1994; Stahl et al., 1994). Once activated, Jak2 kinases phosphorylate several residues on the cytoplasmic domain of the gp130/LIF receptor subunits allowing SH2 (src-homology 2) domain containing proteins to bind. One such protein was identified as STAT 3 (signal transducer and activator of transcription 3) a 'key transcriptional determinant' (Boeuf et al., 1997; Niwa et al., 1998) phosphorylated by Jak2 allowing it to dimerise and translocate to the nucleus where it controls transcription of genes involved in self-renewal. Studies using constitutively active STAT3 mutants found that activation of STAT 3 abrogated the requirement for LIF (Matsuda et al., 1999). Further analysis revealed that one of the mechanisms through which this was achieved was the stabilisation of the transcription factor *c-myc* (Cartwright et al., 2005). Signalling downstream of LIF is not limited to STAT3. The Mitogen Activated Protein Kinase (MAPK) pathway is activated through the SHP-2-Gab-1-Grb-2 complex. Mutation of the SHP-2 tyrosine binding site or inhibition of MEK, a component of the MAPK pathway (see Figure 1-16), with small molecule inhibitors enhances self-renewal thus activation of the MAPK pathway appears to promote differentiation (Burdon et al., 1999).

Although LIF is important for maintaining ES self-renewal it is unable to sustain self-renewal in the absence of FCS and additional extrinsic signals have recently been identified. These include the two serum proteins BMP2/4 (bone morphogenic protein 2 and 4) which are able to act in synergy with LIF in a chemically defined medium to maintain ES cell pluripotency, relieving the requirement for serum (Ying et al., 2003). BMPs activate downstream signalling molecules called Smads (Similar to Mothers Against Decapentaplegic). Smad-4 induces expression of Id (inhibitor of differentiation) transcriptional repressors which repress expression of genes required for neurogenesis, considered as the 'default' differentiation pathway for mouse embryonic stem cells. However, in the absence of LIF, BMP2/4 promotes differentiation, through activation of Smads 1, 5 and 8, towards non-neural lineages demonstrating the fine balancing act required to maintain ES cell self-renewal. Wnt signalling has also been reported to play an important role in the maintenance of self-renewal. Experiments performed with BIO, a small molecule inhibitor of GSK-3

(Glycogen Synthase Kinase-3) downstream of Wnt signalling, or Wnt3a conditioned medium, have demonstrated that activation of the Wnt signalling pathway supports self-renewal and reduces (but does not abrogate) the requirement for LIF (Ogawa et al., 2006; Sato et al., 2004). The use of another small molecule inhibitor, this time LY294002, a PI3K (phosphoinositide-3-kinase) inhibitor, along with dominant negative approaches, has demonstrated the involvement of PI3K-dependent signalling in maintaining optimal self-renewal of murine ES cells (Paling et al., 2004).

1.1.2.3.2 Transcription factors required for maintenance of ES cell self-renewal

As well as the signals mediated through LIF, BMPs, Wnts and PI3Ks, self-renewal is also regulated by a set of transcriptional regulators. The first transcription factor for which a role in self-renewal was identified was the POU domain transcriptional regulator encoded by the *Oct-3/4* gene. Oct-3/4 can activate or repress DNA transcription dependent on the sequences flanking its octamer repeat binding site. It is expressed by undifferentiated ES cells and is down-regulated upon differentiation (Okamoto et al., 1990; Pesce and Scholer, 2001; Rosner et al., 1990; Scholer et al., 1989). Knockout studies have shown Oct-3/4 to be essential for derivation and maintenance of ES cells and for the formation of the pluripotent inner cell mass of the blastocyst (Nichols et al., 1998). The level of Oct-3/4 expression is a critical determinant of cell fate (Figure 1-4). Conditional expression of Oct-3/4 in ES cells, demonstrated that a two-fold increase in expression could cause differentiation towards primitive endoderm and mesoderm lineages. Repression of Oct-3/4 expression meanwhile caused dedifferentiation of ES cells to the trophectodermal lineage (Niwa et al., 2000). Oct-3/4 often binds in partnership with the homeobox domain containing protein transcription factor Sox-2 (SRY (sex-determining region – Y)-related high-mobility-group box protein) which binds to neighbouring sox elements (Pevny and Lovell-Badge, 1997).

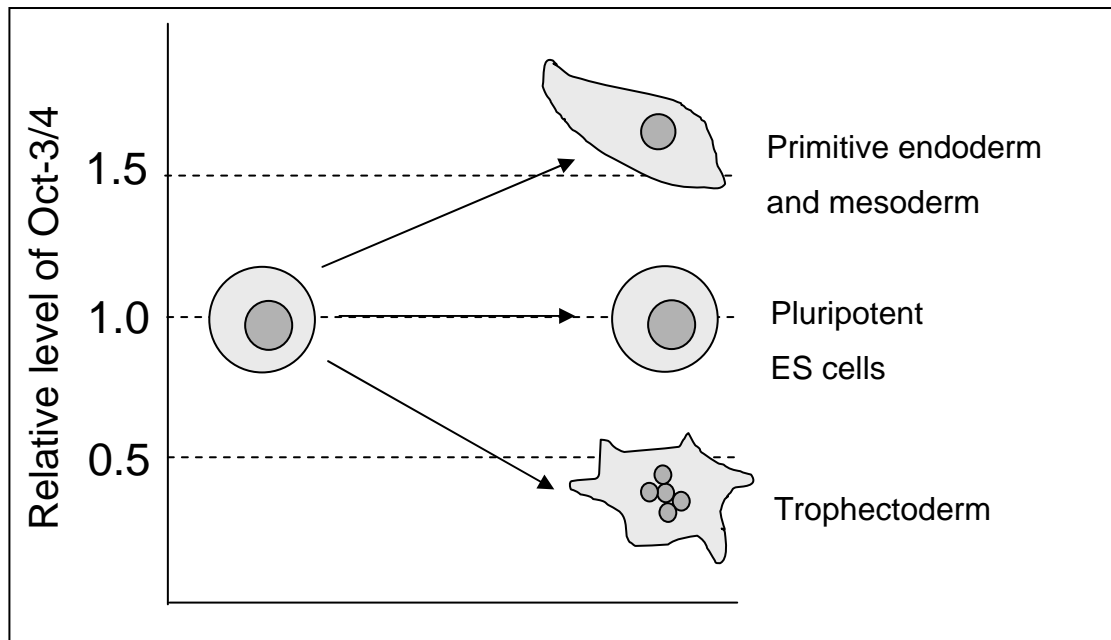


Figure 1-4 Relative expression of Oct-3/4 determines pluripotent cell fate. To maintain pluripotency Oct-3/4 expression must remain within 50% of normal diploid expression. An increase above this directs differentiation towards the primitive endodermal and mesodermal lineages. A decrease below this causes dedifferentiation towards the trophectodermal lineage (reviewed Wobus and Boheler, 2005).

More recently, the homeodomain protein Nanog has been identified as another transcription factor critical for the establishment and propagation of pluripotency in both ES cells and in the epiblast (Chambers et al., 2003; Mitsui et al., 2003; Wang et al., 2003). Overexpression of Nanog can maintain ES cells in a pluripotent state in the absence of LIF and its target genes overlap substantially with those of Oct-3/4 (Loh et al., 2006). The regulators of Nanog are not yet well characterised but recent work has demonstrated that PI3K-dependent signalling regulates Nanog expression through GSK-3 dependent mechanisms (Storm et al., 2007).

Signalling pathways and transcription factor expression/localisation alone cannot complete the self-renewal picture. Epigenetic mechanisms including DNA methylation, histone modification and repression by polycomb group complexes also influence transcription factor mediated self-renewal mechanisms and are thus essential for cell fate decisions (Boyer et al., 2006; Gan et al., 2007; Hattori et al., 2004; Tada and Tada, 2001).

1.1.2.4 Similarities and differences between human and murine embryonic stem cells

Both mouse and human ES cells are derived from the inner cell mass of the pre-implantation blastocyst and can be maintained on a murine embryonic fibroblast (MEF) feeder cell layer. Human ES cells were derived more recently and culture conditions are not currently as well established as for murine ES cells. This may be part of the reason that human ES cell lines have thus far proved less karyotypically stable than their murine counterparts. The most common anomalies, trisomies of chromosomes 12 and 17, appear to enhance growth and self-renewal of human ES cells (Draper et al., 2004). Although differences between human ES cell lines have been reported (Abeyta et al., 2004; Allegrucci and Young, 2006), a recent collaborative study, in which the expression of 17 cell surface antigens and 93 genes was compared between 59 independently derived human ES cell lines from 17 laboratories in 11 countries, demonstrated no significant differences between the cell lines themselves and postulated that reported differences may be due to differences in culture conditions (The International Stem Cell Initiative, 2007).

Many of the well characterised mechanisms of control of self-renewal in mouse ES cells including Wnt signalling and Oct-3/4, Sox2 and Nanog transcription factors are also important in the maintenance of self-renewal in human ES cell lines (Rao, 2004). Oct-4 and Nanog appear to have overlapping binding site in human as well as in mouse ES cells (Boyer et al., 2005). Direct comparison of Oct-4 and Nanog targets identified in human ES cells by Boyer *et al.*, with those identified in murine ES cells indicated limited between species overlap (Loh et al., 2006). Although transcriptome profiling of human and mouse ES cells does indicate key differences between the species (Wei et al., 2005), a recent comparison of the methodology used by each group (ChIP on CHIP analysis from Boyer *et al.*, and ChIP-PET analysis by Loh *et al.*, indicates that observed difference may at least in part be due to methodological differences (Euskirchen et al., 2007).

The most striking difference in regulation of self-renewal between human and mouse ES cells is the inability of LIF to maintain self-renewal of human ES cells. The LIF receptor/gp130 is present at low levels in some human ES cell lines but activation of

the JAK/STAT3 pathway does not appear to be required for human ES cell self-renewal (Ginis et al., 2004; Thomson et al., 1998). A possible explanation for this is may be that the requirement for LIF in mouse ES cells is related to the role of LIF in maintaining the pluripotent state of the inner cell mass during diapause (delayed blastocyst implantation) of which there is no equivalent in the human. Murine LIF knockout embryos are unable to proceed after diapause but in the absence of diapause (which does not occur in the human embryo) the LIF knockout embryo proceeds as normal until gastrulation (Nichols et al., 2001). BMP-4 also has an alternate function in human ES cells, promoting trophectoderm formation rather than ES cell self-renewal (Xu et al., 2002).

In contrast to murine ES cells, FGF signalling appears to play an important role in the self-renewal of human ES cell lines. In the presence of a fibroblast feeder layer or fibroblast conditioned medium 4ng/ml bFGF/FGF2 is sufficient to maintain pluripotency (Amit et al., 2000). Feeder-free ES culture as has been achieved in murine ES cells has not been achieved for many human ES cell lines. However, increased bFGF/FGF2 concentrations (of 100ng/ml) have recently been demonstrated to support human ES cells plated on matrigel (a complex of extracellular matrix proteins) when supported by knockout serum replacement (Levenstein et al., 2006; Xu et al., 2005a; Xu et al., 2005b). Sphingosine-1 phosphate (S-1-P) and Platelet derived growth factor (PDGF) have also been shown to provide a growth signal in serum free culture in the presence of knockout serum replacement (Pebay et al., 2005).

There is still much work to be done in the understanding of both human and murine ES cell self-renewal. The study of mouse ES cells will help inform that of their human equivalents but an appreciation of species differences needs to be maintained.

1.2 Embryonic development and *in vitro* models

1.2.1 Embryonic development – a general overview

Mouse embryonic development is used as a model for the study of mammalian development due to the ease of manipulation of mouse embryos and the ethical-unfeasibility of similar studies in humans. Mouse embryonic development takes place over 20 days, from fertilisation to birth. Figure 1-5 shows the early development of the embryo from fertilisation to gastrulation during which time the three germ layers, mesoderm, endoderm and ectoderm, which later give rise to all tissues and organs of the body, are formed. An overview of early murine development, based upon the following sources is given below (Downs, 2002; Gilbert, 2003; Wild and Fleming, 2002)

1.2.1.1 Pre-implantation development

The fertilised zygote divides through asynchronous rotational cleavage to form a loose ball of 8 cells. This continues dividing and undergoes compaction to form a morula in which cells are tightly packed and stabilised by tight junctions between the outer cells. Cells on the periphery of the day 3 morula endocytose extraembryonic fluid into its centre in a process called cavitation, forming the blastocoel of the blastocyst at day 3.5 post-coitum (E3.5). This creates two distinct cell populations, the inner cell mass (ICM), which goes on to form the embryo proper, and the trophoblast, which forms the embryonic contribution to the placenta, the chorion.

For the first 3.5 days the conceptus is enclosed in the zona pellucida (originally the extracellular matrix of the egg) which prevents implantation before the conceptus has reached the uterus. The blastocyst now ‘hatches’ from the zona pellucida using a trophoblastic protease, stryptsin, to lyse a hole which the growing blastocyst then squeezes through. The first segregation of the inner cell mass at E4-4.5 creates the hypoblast, (or primitive endoderm) which delaminates to line the blastocoel cavity and the epiblast (or primitive ectoderm) from which the embryo proper is formed (see Figure1-5, E4-4.5blastocyst).

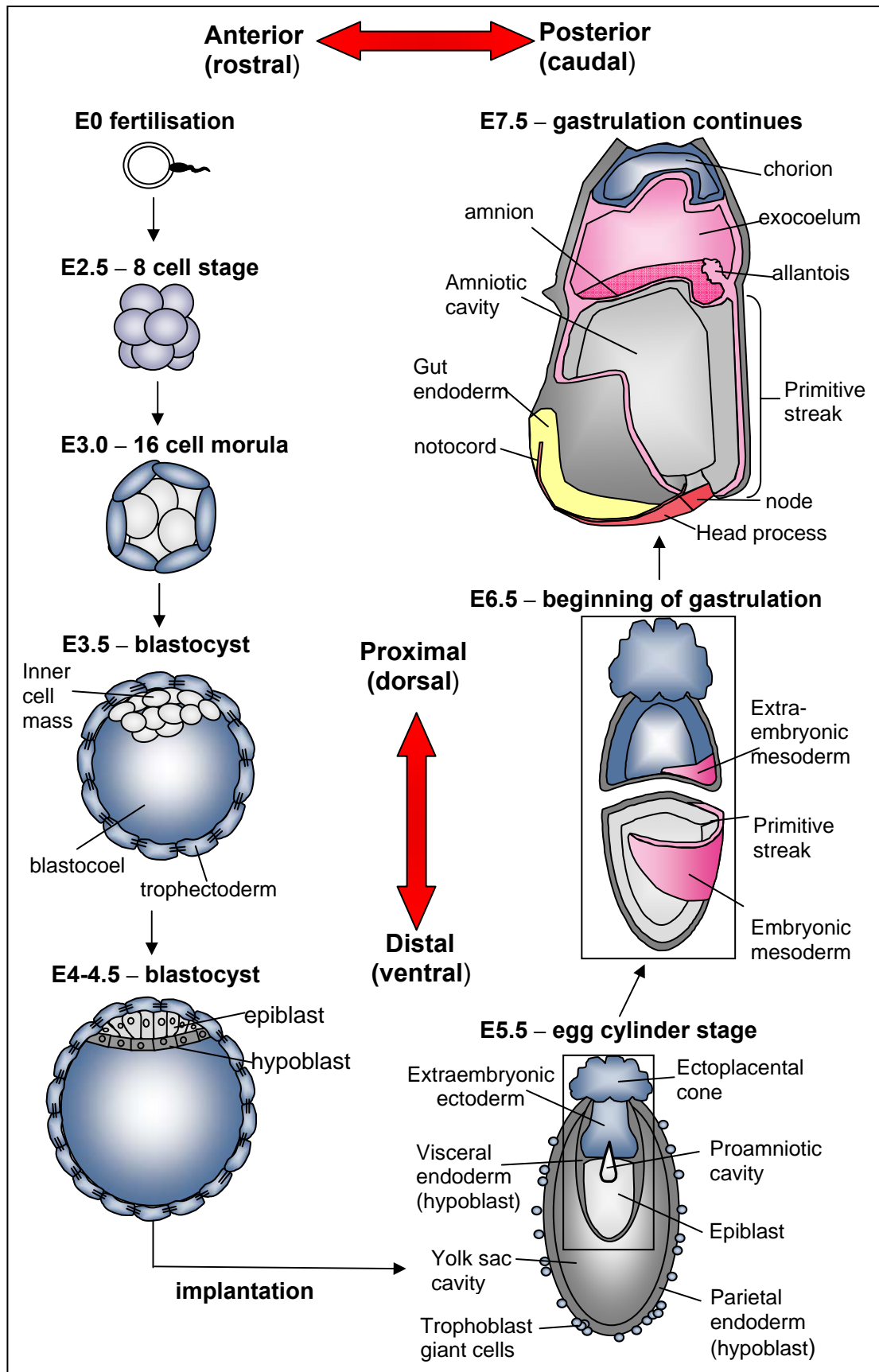


Figure 1-5 Mouse embryonic development. Diagram adapted from Wild and Fleming 2002 and Gilbert, 2003. From E5.5 onwards the yolk sac, placental trophoblastic lineages and parietal endoderm are excluded for clarity.

1.2.1.2 Implantation

Implantation occurs at E4.5 and over the next 24 hours the polar trophoectoderm/trophoblast (proximal) proliferates down into the blastocyst forcing the epiblast into the blastocoel. The hypoblast further differentiates and migrates to line the blastocoel. Hypoblast still adjacent to the epiblast forms the visceral (or proximal) endoderm whilst the hypoblast no longer in contact with the epiblast becomes the parietal (or distal) endoderm. The parietal endoderm associates with the giant trophoblastic cells of the mural trophoblast and secretes a thick basement membrane converting the blastocoel into the primary yolk sac. The yolk sac becomes filled with maternal plasma proteins from polar trophoectoderm infiltration into maternal capillaries. The visceral endoderm digests these proteins to provide nutrients for the rapidly dividing epiblast which has become organised into a pseudostratified epithelium and also induces apoptosis of central cells in the epiblast forming the proamniotic cavity. At E5.5 the conceptus is at the egg cylinder stage illustrated in Figure 1-5, E5.5-egg cylinder stage.

1.2.1.3 Gastrulation

Gastrulation begins at E6.5 with the formation of the primitive streak which begins as a thickening of the epiblast at the posterior end of the embryo where the epiblast joins the visceral endoderm. As soon as the streak is formed epiblast cells begin to migrate through it. Cells emerging from the primitive streak come to lie between the outer epiblast and the visceral endoderm. The first of these form mainly mesoderm, both extraembryonic and embryonic (Figure 1-5, E6.5 – egg cylinder formation). Mesoderm destined for extraembryonic fates migrates proximally to form the membranes of the allantois, chorion and amnion (Lawson et al., 1991). The amniotic folds fuse to form the exocoelomic cavity. Migrating mesodermal aggregates destined to differentiate into blood islands colonise the presumptive yolk sac and eventually mesoderm lines the cavity forming the lining of what has become the visceral yolk sac (Ferkowicz and Yoder, 2005). Embryonic mesoderm migrates distally forming structures such as the somites and lateral plate mesoderm (Lawson et al., 1991). As development progresses the streak extends distally, recruiting more delaminating epiblast cells, to the late streak stage at which it reaches the distal tip of the embryo. Cells emerging from the streak now form both mesoderm and endoderm

(Figure 1-5 E7.5 – gastrulation continues). Ectoderm forms from epiblast cells which do not migrate through the streak and relies upon interaction with the mesoderm for its further development. Lineage specification of ectoderm, mesoderm and endoderm is illustrated in Figure 1-6.

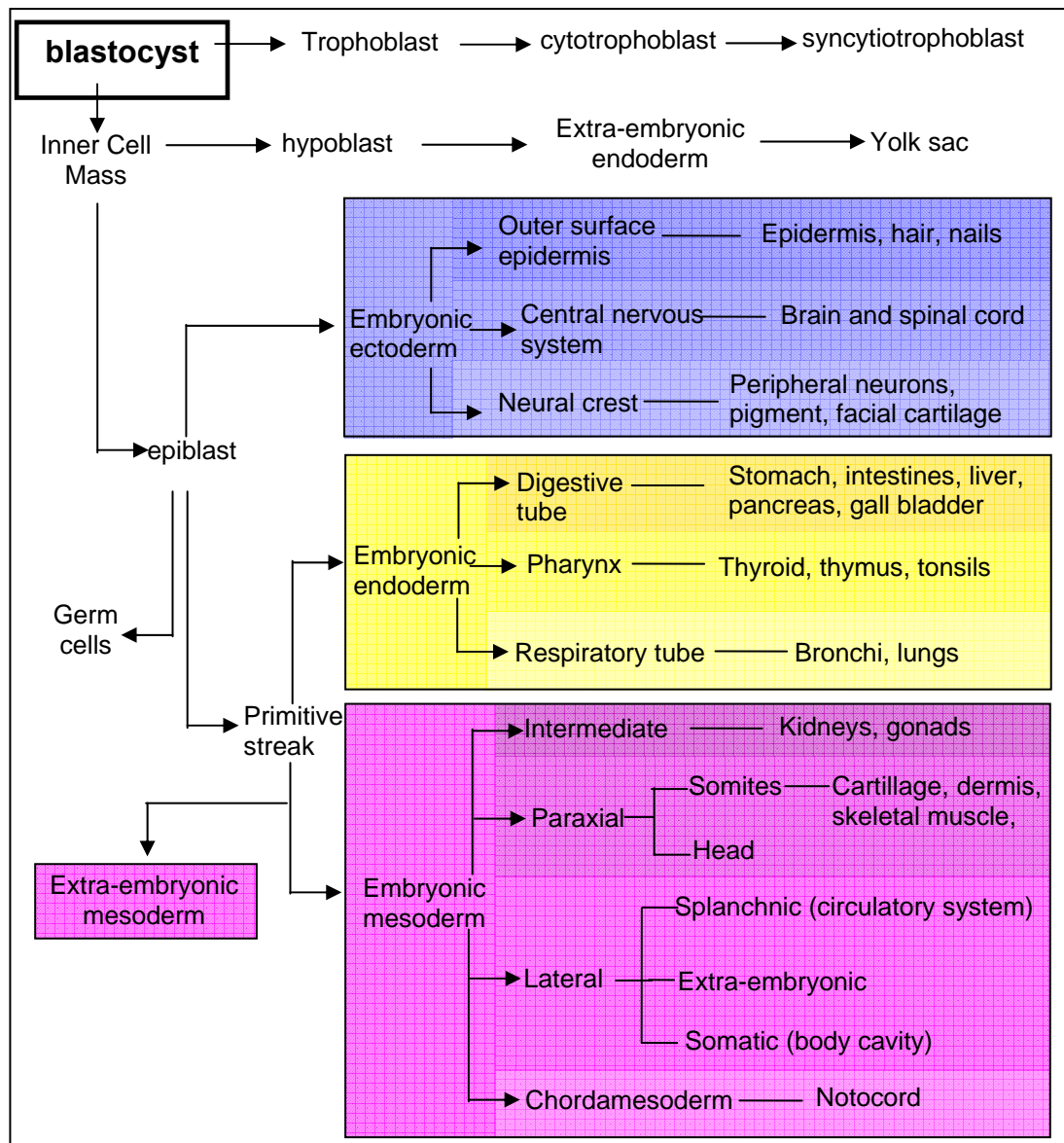


Figure 1-6. Lineage specification of the primary germ layers in the mammalian embryo. Diagram adapted from Gilbert, 2003

By E7.5 the streak has extended to the distal tip and the node forms from an area of cells of mesodermal origin at the end of the streak with no covering of visceral endoderm and from the epiblast above them. The node is the area from which the head is formed and it also contributes to the neural tube and axial mesoderm.

Definitive gut endoderm arises from the same area. The extra-embryonic mesoderm, which migrated through the primitive streak at the beginning of gastrulation, has expanded and spread posteriorly. Cavities in this extra-embryonic mesoderm have coalesced to form exocoelom and the allantois, amnion and chorion contributions have been specified. Embryonic mesodermal precursors have migrated into the yolk sac and formed the blood islands which will generate the first vascular endothelial and primitive haemopoietic cells (Kinder et al., 1999).

1.2.2 Modelling embryonic development *in vitro*

As discussed in 1.1.2 mouse ES cells have the capacity to form any cell of the three germ layers under appropriate conditions. This property allows their exploitation as a model of development to investigate lineage commitment. There are several advantages to this *in vitro* approach over studies performed in the whole embryo. *In vitro* differentiation can provide a means to gain access to large populations of precursor or lineage-committed cells relatively easily, providing suitable quantities of material for protein and RNA work. The ES cell model can also be used in gene-targeting approaches where *in vivo* studies may be more difficult or not possible due to, for example, embryonic lethality. In addition, particularly with regard to human ES cells, it provides a more ethically robust mode of study.

The most common method of differentiating ES cells is by removing LIF and placing them in an environment conducive to the formation of embryoid bodies (EBs) (Doetschman et al., 1985; Keller, 1995). Embryoid bodies are three dimensional aggregates of embryonic stem cells the development of which mimics early embryogenesis (see Figure 2-1 for pictures). They can be formed in one of three ways (Keller, 1995):

1. Embryonic stem cells grown in methylcellulose semi-solid medium at 1×10^4 /ml for at least three days prior to study.
2. Embryonic stem cells cultured in hanging drops of media containing 400-1000 cells for 2 days and then replated into a suspension culture.
3. Embryonic stem cells grown on non-adhesive culture plates.

The other main methods of ES cell differentiation are differentiation on a stromal cell feeder layer (e.g. OP-9) (Nakano et al., 1994) or differentiation on extracellular

matrix proteins such as collagen (Nishikawa et al., 1998a). The method used (and the growth factors added) influences the balance of cell lineages formed. Formation of embryoid bodies in semi-solid medium in the presence of the appropriate cytokines, or differentiation on a feeder layer of OP-9 cells biases differentiation towards mesodermal lineages including haemopoietic, endothelial and muscle (Dang et al., 2002; Desbaillets et al., 2000). Further analysis of developing populations within embryoid bodies, on feeder layers or in adherent differentiation, may require replating of the differentiated populations into new culture conditions e.g replating embryoid bodies formed in hanging drops to direct cardiac differentiation (Sachinidis et al., 2003a) or replating embryoid bodies formed in methylcellulose into a haemopoietic colony assay to analyse the haemopoietic progenitors present (Keller et al., 1993). Additionally, analysis of differentiated ES cell populations can be conducted morphologically, genetically, biochemically or immunologically (Keller, 1995).

Embryoid bodies have proved good models of embryogenesis being able to differentiate into the three germ layers and then into more committed cell types, for example; cardiomyocytes, skeletal muscle, endothelial cells, neuronal cells, adipocytes and haematopoietic precursors, mimicking development of the embryo (Desbaillets et al., 2000). Study of genetic markers of embryonic differentiation and their appearance in EBs supports the observation that EB development mimics embryonic differentiation both spatially and temporally. After differentiation is initiated an endodermal outer layer differentiates within the embryoid body. This is followed by development of an ectodermal 'rim' and mesodermal specification (Wobus and Boheler, 2005). Days 3.5 to 4.5 in the EB represent the gastrulation stage which occurs *in vivo* between day 6.5 and 7.5 and after day 6 EBs are equivalent to the early organogenesis stage which occurs from day 7.5 onwards in the embryo (Leahy et al., 1999). Disruption of primary EBs allows expansion of any one of the three germ layers preferentially in a further culture system enabling dissection of the development of different systems. One example of this is the dissection of the development of haemopoietic and endothelial cell lineages. The BL-CFC (Blast Colony-Forming Cell) is thought to be the *in vitro* equivalent of the haemangioblast, defined as a common precursor of haemopoietic and endothelial lineages (see section 1.4.2). The BL-CFC population arises at around Day 3 in

primary embryoid bodies. Dissociation of primary embryoid bodies at this point allows expansion and differentiation of this BL-CFC population in a secondary blast assay which allows further dissection of the formation of haemopoietic and endothelial progenitors (Faloon et al., 2000; Kennedy et al., 1997). Cells are plated into fresh methylcellulose medium containing Vascular endothelial growth factor (VEGF) and conditioned medium from D4T embryoid body-derived endothelial cells to promote formation of blast colonies which reportedly contain both haemopoietic and endothelial progenitors (Choi et al., 1998; Faloon et al., 2000; Kennedy et al., 1997).

1.2.3 Haemopoietic and vascular development

In the adult, as well as the developing embryo, the haemopoietic and vascular systems play important inter-connecting roles. The importance of the circulatory system is demonstrated by its early requirement in the embryo. By E10 diffusion (see 1.2.1 embryonic development – a general overview) is no longer sufficient to supply nutrients to or remove waste from the developing embryo and a functioning vascular system must be in place to ensure embryonic survival (Fujiwara et al., 1996; Porcher et al., 1996; Shalaby et al., 1995). The importance of vascularisation is such that that the earliest mesodermal derivatives to differentiate are the endothelial and haemopoietic precursors which form the primitive vascular system (Kinder et al., 1999).

1.2.3.1 Establishment of primary circulation (including primitive haemopoiesis)

The first haemopoietic cells to arise are primitive erythrocytes, along with small numbers of megakaryocytes and macrophages, although it is unknown whether these cells derive from a common site within the yolk sac. Primitive erythrocytes are larger than foetal and adult erythrocytes and contain an embryonic form of haemoglobin (β -H1-globin) along with the adult form (β -major-globin) (Leder et al., 1992). They are nucleated until between E12.5 -16.5 when enucleation, thought to be associated with terminal differentiation, occurs (Fraser et al., 2007; Kingsley et al., 2004). Both the endothelium of blood vessels and the haemopoietic cells themselves are derived from the blood islands which form in the proximal visceral yolk sac (furthest from the

embryo) when aggregates of mesodermal cells (thought to have already undergone lineage commitment (Huber et al., 2004; Palis et al., 1999)) colonise the presumptive yolk sac at around E7 to form the blood islands (Haar and Ackerman, 1971; Palis et al., 1995). It would be expected that the vascular endothelium and haemopoietic precursors would differentiate simultaneously and proximally to form a functional vascular system and in the 12 hours following mesodermal aggregation the inner cells of the blood islands differentiate into maturing primitive erythrocytes whilst the peripheral cells form endothelium (Haar and Ackerman, 1971). This observation of proximal development was first made in the chick blastodisc by Florence Sabin (Sabin, 1920) and gave rise to the theory that both lineages differentiate from a common precursor, the haemangioblast (Murray, 1932). The existence of the haemangioblast has been a cause of much controversy and will be discussed in more detail in section 1.2.3 The Haemangioblast.

By E8, at which point somatogenesis begins, the endothelial cells of the blood islands have anastomosed to form the capillary network or vascular plexus which then develops asymmetrically in the visceral yolk sac whilst remaining centred round the blood islands. At the same stage, primitive erythrocytes remain restricted to the blood islands. Simultaneously, in the embryo, angioblasts (the precursors of endothelial cells) begin to differentiate and coalesce forming the aortae, the first blood vessels of the embryo proper which form initially in the absence of associated haemopoietic precursors (Jaffredo et al., 1998). By E8.25 the vascular plexus has spread throughout the yolk sac and formed vitelline connections with the vasculature of the embryo proper. It is around this time that small numbers of erythrocytes are first observed away from the blood islands in the distal yolk sac and the embryo proper, and that the first true contractions of the heart as an organ are observed (Ji et al., 2003; McGrath et al., 2003).

Between E8.5 and 10.5 the numbers of primitive haemopoietic progenitors in the yolk sac expands exponentially (Ji et al., 2003) and continue to infiltrate the maturing vascular system. By E9.5 the vascular system has matured into an arborized network formed by both vasculogenesis and angiogenesis. A fully functional circulation is established by E10.5 with the vascular network fully populated with maturing primitive erythrocytes which now comprise 40% of the embryo (McGrath et al.,

2003). This correlates well with the timing of fatality in mouse mutants deficient in formation of any part of the circulatory system (Fujiwara et al., 1996; Porcher et al., 1996; Shalaby et al., 1995; Wakimoto et al., 2000).

1.2.3.2 *Definitive haemopoiesis*

Definitive haemopoiesis gives rise to all definitive erythroid, myeloid and lymphoid lineages which arise from a common haemopoietic stem cell. Until E10.5 yolk sac haemopoiesis is the only source of blood in the developing embryo. At this point the progeny of definitive haemopoiesis begins to emerge in the circulation. Definitive erythrocytes are fully matured before entering the circulation. They are also smaller, and utilise some different transcriptional machinery and growth factor signalling (e.g. c-kit is essential for definitive but not primitive haemopoiesis; (McGrath and Palis, 2005) than their primitive counterparts. The number of circulating definitive haemopoietic cells increases from E11.5 onwards and they become predominant between E13.5 and E14.5 (Kingsley et al., 2004). Sites of early haemopoiesis in both human and mouse are illustrated in Figure 1-7 below.

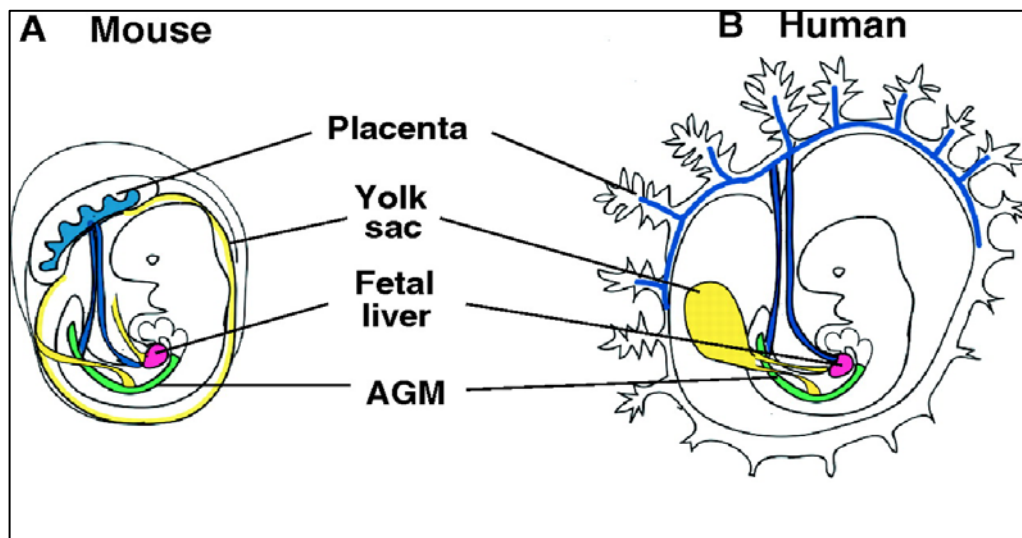


Figure 1-7 An illustration of haemopoietic organs in (A) a mouse and (B) a human embryo, at 11 days and 5 weeks of gestation, respectively (yellow, yolk sac; green, dorsal aorta of the aorta-gonad-mesonephros (AGM) region; red, fetal liver; blue, umbilical vessels and fetal vasculature in the placenta). The yolk sac in the mouse embryo surrounds the embryo due to axial rotation, an anatomical peculiarity of mice. In humans and other mammals the yolk sac is balloon shaped and connected to the mid-gut via a long stalk. The hematopoietic function of the human placenta has not yet been experimentally proven. Diagram from Mikkola 2006.

The site of initiation of definitive haemopoiesis is still some-what controversial. In part this is due to differing definitions of the term. Some researchers define 'definitive haemopoiesis' as the formation of all haemopoietic lineages, excluding primitive erythrocytes and early macrophages (and megakaryocytes) (Keller et al., 1999; McGrath and Palis, 2005). Others define it as the expansion and differentiation of the definitive haemopoietic stem cell (HSC), HSCs themselves being defined as "those cells possessing their own ability to differentiate and expand on definitive, adult, hematopoietic territories" (Medvinsky and Dzierzak, 1999).

Using the first definition, definitive erythroid and myeloid precursors have been found in the yolk sac as early as E8.25. They are not thought to mature *in situ*, rather migrating to the presumptive foetal liver where they engraft and mature (Palis et al., 1999). The second site of definitive haemopoiesis is the para-aortic splanchnopleure (P/Sp E8.5-9.5) /aorta-gonad-mesonephros (AGM E10.5-11.5) region which contains the dorsal aorta, genital ridge/gonads, and pro/mesonephros area and which gives rise to all haemopoietic lineages. The exact origin of haemopoietic cells in this region is unclear, though the close anatomical relationship between haemopoietic clusters and endothelial cells in the dorsal aorta has led to postulation that the blood cells of the AGM differentiate from a haemogenic endothelium (see section 1.2.4.4). The AGM is also well established as the site of emergence of HSC capable of reconstituting an adult recipient (de Bruijn et al., 2000; Medvinsky and Dzierzak, 1996) with HSC emerging at around E10.5. HSCs with the capacity to rescue a lethally irradiated adult mouse have not been isolated prior to this developmental stage, although HSCs capable of repopulating the newborn (possibly through maturation in the foetal liver which is still capable of limited haemopoiesis at birth), but not the adult, are found much earlier at E9 both in the P/Sp and at greater frequency in the yolk sac (Yoder et al., 1997). More recent work using an elegant Runx1 labelling method to label haemopoietic cells in the yolk sac at E7.5, prior to their emergence in the embryo proper, has suggested at least a partial yolk sac contribution to HSCs and haemopoiesis in the adult mouse via colonisation of the umbilical vein, AGM and foetal liver (Samokhvalov et al., 2007). However, as the Cre-loxP-Runx-1 labelling method is reliant on expression of Runx-1 at E7.5 occurring exclusively in the yolk sac (which has not been proven) this work cannot be considered proof of a yolk sac contribution.

HSCs have also been located in the embryonic contribution to the placenta, which appears to constitute a site of HSC origin (Ottersbach and Dzierzak, 2005; Zeigler et al., 2006), and in the umbilical and vitelline arteries (de Bruijn et al., 2000). By E10 definitive haemopoiesis in the foetal liver, which is seeded by cells from both the yolk sac and AGM, has begun. This becomes the major site of foetal haemopoiesis until just before birth when haemopoiesis in the bone marrow, the main site of adult haemopoiesis, becomes dominant (Baron, 2003; Mikkola and Orkin, 2006) as illustrated in Figure 1-8.

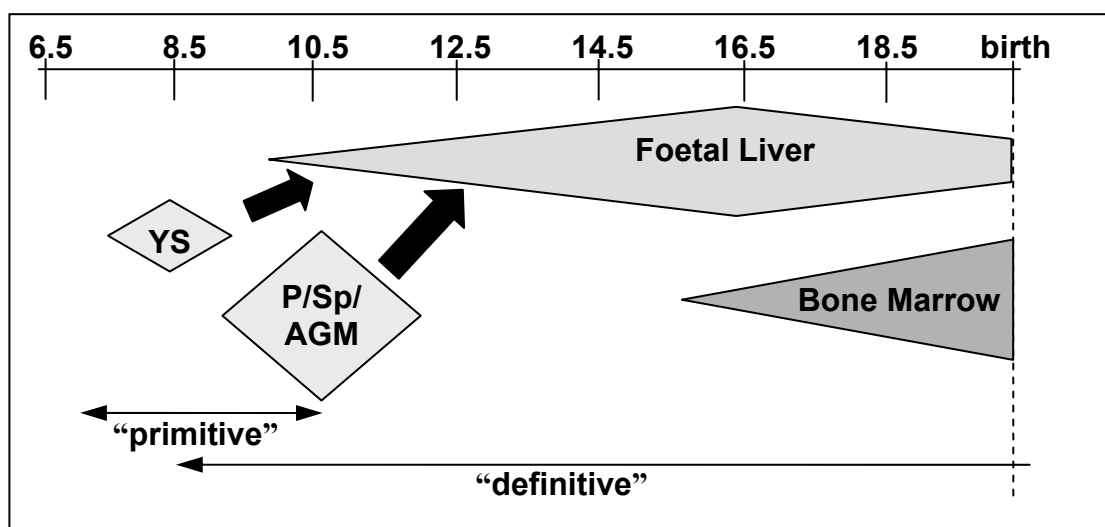


Figure 1-8 Changes in the major haemopoietic sites during development. YS-yolk sac, P/Sp/AGM-Para-aortic-Splanchnopleure/Aorta-Gonad-Mesonephros, Diagram adapted from Baron, 2003.

1.2.4 The Haemangioblast

1.2.4.1 The origins of the haemangioblast hypothesis

The haemangioblast can be defined as a common precursor for haemopoietic and endothelial cells, particularly in the early embryo. The first endothelial and haemopoietic cells develop in the blood islands, aggregates of mesodermal cells which migrate into the extra-embryonic visceral yolk sac during gastrulation (see also sections 1.2.1 – ‘Embryonic Development – a general overview’ and section 1.2.3 ‘Haemopoietic and Vascular Development’). The inner cells of these blood islands differentiate into primitive haemopoietic cells whilst the outer cells

differentiate into endothelial cells which later fuse with other blood islands to begin to form the yolk sac blood vessels. The developmental proximity of these two lineages led to the first proposal of a common precursor, the haemangioblast, by Murray in 1932 (Murray, 1932) illustrated in Figure 1-9.

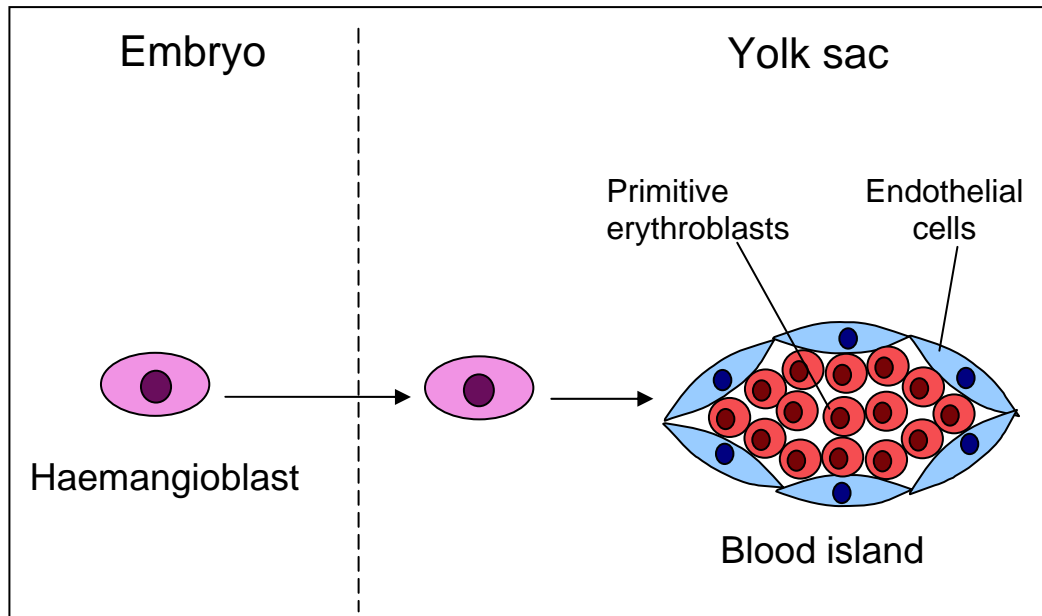


Figure 1-9 Historical model of the haemangioblast. Mesodermal cells migrating out of the primitive streak colonise the yolk sac. One clonal haemangioblast forms one blood island proliferating to form an aggregate the inner cells of which differentiate to form the blood whilst the outer cells form the endothelium.

1.2.4.2 In vitro studies support the existence of the haemangioblast

The existence of the haemangioblast is supported by gene expression data which has demonstrated expression of a number of different genes to be common to both haemopoietic and endothelial lineages including Foetal liver kinase-1 (Flk-1) (Kabrun et al., 1997; Millauer et al., 1993), the stem cell leukaemia gene (*Scl*) (Visvader et al., 1998), *Cd34* (Fina et al., 1990), *erythropoietin* (Anagnostou et al., 1994) and *c-kit* (Bernex et al., 1996). Indeed, in recent years ever more endothelial markers including VE-cadherin (Vascular Endothelial-cadherin) (Kim et al., 2005) and Pecam-1 (platelet/endothelial cell adhesion molecule-1) (Baumann et al., 2004) have been identified in haemopoietic populations leaving very few true endothelial-specific markers (Fleming, 2005). A number of genes including Flk-1 (Shalaby et al., 1995) and *Tgf- β 1* (Dickson et al., 1995) in the mouse and *Cloche* in *Danio rerio*

(zebrafish) (Stainier et al., 1995) affect both lineages when knocked-out, further supporting a common origin hypothesis. Though these results support the haemangioblast hypothesis, the existence of the haemangioblast *in vivo* has been more difficult to prove.

In vitro studies, using the differentiation of mouse ES cells into embryoid bodies as a model of embryogenesis, have identified a transient cell population which arises after 2.5-5 days of culture, prior to haemopoietic commitment, which is termed the Blast colony forming cell (BL-CFC). Upon culture in methylcellulose, with the addition of VEGF and conditioned medium from the D4T embryoid body-derived endothelial cell line, the BL-CFC gives rise to blast colonies containing both haemopoietic and endothelial cells and thus represents the *in vitro* haemangioblast (Choi et al., 1998; Kennedy et al., 1997). The clonal origin of these haemopoietic and endothelial lineages was demonstrated using two labelled embryonic stem cell lines in mixing studies (Choi et al., 1998) and work by Fehling and colleagues demonstrated the haemangioblast to be derived from cells in the culture which co-expressed the tyrosine kinase receptor Flk-1 and the T-box gene, *Brachyury* (see section 1.3.1) (Fehling et al., 2003).

1.2.4.3 The haemangioblast in vivo

Although molecular and *in vitro* studies support the existence of the haemangioblast it has been more difficult to study in the actual embryo. Huber *et. al.* recently identified a cell *in vivo* which first appears in the primitive streak at the mid-streak stage, coexpresses Flk-1 and *Brachyury* and which when cultured *in vitro* behaves in the same way as BL-CFC, forming blast colonies containing both haemopoietic and vascular endothelial cells (Huber et al., 2004). This work, the first to identify a cell with haemangioblastic potential *in vivo*, indicates that vascular and haemopoietic commitment begins prior to migration of these cells into the yolk sac. Other research into yolk sac, definitive and adult haemopoiesis and vasculogenesis has added further complexity to the haemangioblast model and raised new questions.

Recent research into yolk sac haemopoiesis and vasculogenesis *in vivo* has begun to cast doubt upon the haemangioblastic origin of *all* yolk sac vascular endothelium. Vascular development without associated haemopoiesis has been observed at sites

away from the blood islands (McGrath et al., 2003) and recent co-culture experiments, using cells isolated from embryos at various stages observed endothelial progenitors earlier than progenitors of the haemopoietic lineages (Furuta et al., 2006). Further support for the existence of a second more committed endothelial precursor population has been gained from recent lineage tracing experiments (Ueno and Weissman, 2006).

1.2.4.4 Haemogenic endothelium

Definitive haemopoiesis in the AGM region also develops in close association with endothelial cells. Molecular characterisation of AGM haemopoietic stem cells revealed expression of endothelial markers including VE-Cadherin (Taoudi et al., 2005) and Flk-1 (Jaffredo et al., 1998), which are later down-regulated, supporting a lineage relationship. A close anatomical relationship between clusters of haemopoietic cells and endothelial cells lining the ventral floor of the dorsal aorta (reviewed Dieterlen-Lievre et al., 2006) and the formation of the aorta and vitelline arteries at least one day prior to the appearance of haemopoietic progeny in the embryo proper, (Jaffredo et al., 1998) has led to the hypothesis that the blood cells of the AGM region differentiate from a ‘haemogenic endothelium’ of the dorsal aorta.

Cell sorting studies have demonstrated that cells of AGM origin display a plethora of endothelial markers including Pecam-1, CD34, Flk-1 and VE-cadherin, have the ability to take up Acetylated Low Density Lipids (AcLDL) and are able to generate CD45 expressing haemopoietic cells including lymphocytes *in vitro* (Nishikawa et al., 1998b; Oberlin et al., 2002). In labelling studies, using intra-cardiac injection to label the endothelial lining of blood vessels in both chick and mouse embryos with either AcLDL or a non-replicative retroviral vector containing a reporter gene, both CD45-expressing budding cluster cells and definitive erythrocytes in the blood stream were found to be labelled. Dissociation of the AGM region, immediately following labelling, demonstrated that the labelled cells expressed other endothelial markers and had the ability to differentiate into haemopoietic lineages *in vitro* (Jaffredo et al., 1998; Jaffredo et al., 2000; Sugiyama et al., 2003). Further supporting evidence for the haemogenic endothelium came from studies in Ly6A-GFP mice in which GFP positive cells confirmed as HSCs by adoptive transfer were

found in the endothelial wall of the ventral floor of the dorsal aorta (de Bruijn et al., 2002).

Haemogenic endothelium is both spatially and temporally restricted to the ventral floor of the dorsal aorta from around E9.5, peaking at E11.5 and declining thereafter (Yao et al., 2007). Studies using chick/quail chimeras have suggested that haemogenic endothelium lining the ventral floor of the dorsal aorta is of splanchnopleural origin whereas the rest of the dorsal aorta at that time is of somatic origin. As haemogenic endothelium differentiates and migrates into the blood stream somatic endothelium, which is non-haemogenic, replaces it to maintain vessel integrity (Pouget et al., 2006). This study may explain both the temporal and spatial restriction of haemogenic endothelium and is supported by further studies in both quail/chick and quail/mouse chimeras which suggests that vessel formation in splanchnopleural tissues of the embryo occurs *in situ* whereas vessel formation in somatic tissues requires immigration of angioblasts (Pardanaud et al., 1989; Pudliszewski and Pardanaud, 2005).

The existence of haemogenic endothelium in the AGM is by no-means proven. Similar evidence has been interpreted as indicative of an AGM haemangioblast (Yao et al., 2007) (though there is no proof of balanced bipotentiality), whilst other studies point to sub-endothelial mesenchymal patches as the origin of the haemopoietic clusters (Bertrand et al., 2005; North et al., 2002). Still greater developmental potential has been postulated for these endothelial cells with some evidence of myogenic differentiation ability (Tavian et al., 2005). The endothelial lining of the floor of the dorsal aorta obviously has an important role to play in definitive haemopoiesis but little is known about the origin (haemangioblastic?) of these haemopoietic endothelial cells.

1.2.4.5 The haemangioblast in the adult organism?

Whilst endothelium appears to play an important role in definitive haemopoiesis in the embryo, in the adult organism there is evidence that HSCs are involved in neovascularisation and maintenance of the vascular system. *In vitro* differentiation of enriched populations of haemopoietic stem cells and monocytes towards the endothelial lineage has been demonstrated (Asahara et al., 1997; Fujiyama et al.,

2003; Zhang et al., 2005) but phenotypic overlap between monocytes with angiogenic potential and vascular endothelial cells has complicated *in vitro* studies (Rohde et al., 2006; Schmeisser and Strasser, 2002). *In vivo* experiments in myeloablated mice with donor bone marrow have demonstrated engraftment of endothelial cells of donor origin both in response to injury and in tissue homeostasis. The donor cells contain no karyotypic anomalies suggesting that the cells are not the result of fusion of a donor haemopoietic cell with an existing host endothelial cell (Bailey et al., 2004; Elsheikh et al., 2005; Grant et al., 2002). Contribution of donor-derived endothelial cells has also been observed in human patients receiving sex mismatched bone marrow transplants, both in vessel homeostasis and in tumour angiogenesis (Jiang et al., 2004; Peters et al., 2005). Human endothelial cells have also been found to participate in neovascularisation of ischaemically injured retinas in NOD/SCID mice previously rescued with human Umbilical Cord Blood (UCB) after myeloablation (Cogle et al., 2004).

It is well known that a single HSC has the ability to reconstitute the haemopoietic potential of myeloablated mice (Osawa et al., 1996) and three groups have demonstrated this single cell approach to also be successful in providing a donor contribution to the endothelium of a recipient animal (Bailey et al., 2004; Grant et al., 2002; Larrivee et al., 2005). This evidence supports the haemangioblast hypothesis in the demonstration of a clonal origin for both haemopoietic and endothelial cells in the recipient animal but does not preclude the possibility that the cell responsible is in fact a less committed multipotent stem cell with the ability to differentiate into multiple tissue types (reviewed Kucia et al., 2005).

The potential therapeutic benefits of being able to stimulate neovascularisation in conditions such as myocardial infarction, cerebrovascular accident and wound healing are obvious. However, the contribution of bone marrow-derived cells to neovascularisation appears to constitute only a small part of the involvement of bone marrow in vascular growth. The paracrine stimulation of vascularisation seems of even greater importance and is therefore the main area of research in the application of bone marrow-derived cells in vascular therapy (reviewed Cho et al., 2006; Schatteman et al., 2007; Werner and Nickenig, 2006).

1.2.4.6 The current state of the haemangioblast hypothesis

In recent years, extensive research into the identity and role of the haemangioblast at different developmental stages and in distinct tissue compartments appears to have further confused, rather than clarified, the field. Ueno and Weissman recently published work disproving the original hypothesis of a clonal haemangioblast giving rise to a single blood island (illustrated in Figure 1-9). Their work, using a novel system in which 3 ES cell lines, expressing different fluorescent markers, were injected into wild type embryos to create chimeras, found more than one colour fluorescence to be present in individual blood islands in chimeric animals proving that the blood islands themselves are not clonally derived from a single haemangioblastic cell (Ueno and Weissman, 2006). As the investigators had used *three* coloured ES cell lines to produce the chimeras the chimerism in the blood islands could provide more clues to the origin of its constituent cells.

The possible outcomes of the experiment are illustrated in Figure 1-10. Most blood islands in the study belonged to type IV in which either;

- A) there were no haemopoietic cells exhibiting fluorescence of a colour which was not also seen in the endothelial population
- B) there were no endothelial cells exhibiting fluorescence of a colour not also found in the haemopoietic population
- C) Both haemopoietic and endothelial populations contain cells exhibiting fluorescence of more than one colour, but no haemopoietic cell is the same colour as an endothelial cell (and vice versa).

The rest of the blood islands fell into type II in which any colour of fluorescence found in the blood island was exhibited in cells of both lineages.

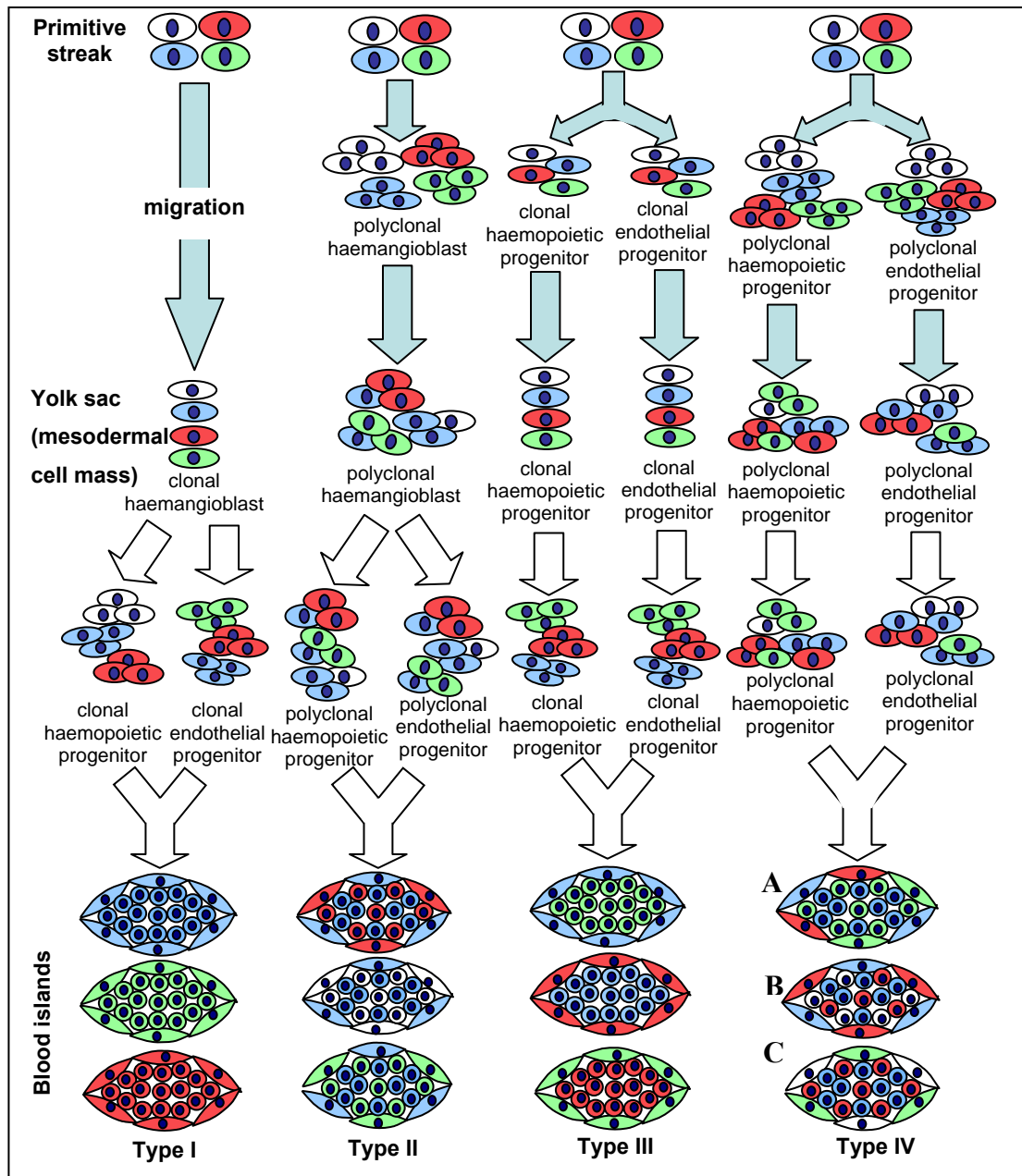


Figure 1-10 Schematic of expected chimerism in yolk sac blood islands in the Ueno/Weissman study (Ueno and Weissman, 2006). Type I blood islands comprised of cells of a single colour would occur if one bipotential haemangioblast gave rise to each blood island. Type II blood islands in which both endothelial and haemopoietic cells are chimeric but share the same mixture of colours would occur if bipotential progenitors proliferated in the primitive streak and mixed during migration. If endothelial and haemopoietic progenitors were generated separately in the primitive streak and each blood island was formed from one of each progenitor blood islands in which all endothelial cells and all haemopoietic cells were a single colour would form giving rise to either type III or type I blood islands. Lastly if endothelial and haemopoietic progenitors were generated separately in the primitive streak and each blood island was derived from several of each progenitor type IV blood islands

would arise. Type IV can be sub classified into A-C. If the fluorescence of the haemopoietic cells in a particular blood island was a subgroup of that found in the endothelial cells, they are defined as A. If the endothelial cells are a subgroup of those in the hematopoietic cells, they are type B. If some colours do not overlap in both lineages, they are classified as type C.

The investigators interpreted these results to support one of the following two hypotheses;

- A) the haemangioblast is the common progenitor of both haemopoietic and endothelial lineages but does not always divide asymmetrically. In this hypothesis one haemangioblast cell could form either purely haemopoietic progenitors, purely endothelial progenitors or both haemopoietic and endothelial progenitors (Figure 1-11A)
- B) the haemangioblast constitutes some of the haemopoietic and endothelial capacity of the blood islands and is augmented by endothelial and/or haemopoietic specific progenitors not themselves derived from the haemangioblast (Figure 1-11B) (Ueno and Weissman, 2006).

Work by Furuta *et al.*, in the mouse and Vogeli *et al.*, in zebrafish also support hypothesis B in which endothelial and haemopoietic precursors exist in addition to the bipotential haemangioblast (Furuta et al., 2006; Vogeli et al., 2006).

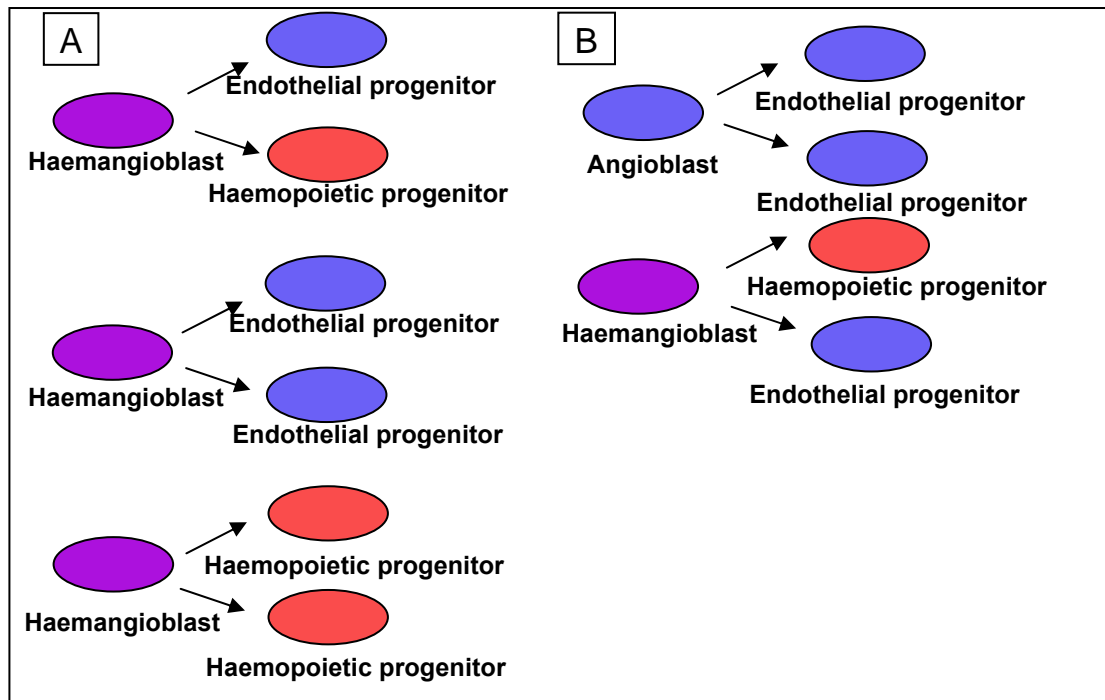


Figure 1-11 Current haemangioblast hypotheses as put forward by Ueno and Weissman, 2006. Model A. Bipotential haemangioblast model. One haemangioblast can differentiate into either endothelial, haemopoietic or both lineages. Model B. The haemangioblast and endothelial specific progenitor model. Haemopoietic and endothelial cells are derived from the haemangioblast and endothelial cells can also be derived from an endothelial specific progenitor the angioblast. A progenitor specific for haemopoiesis may also exist.

The definition of the haemangioblast appears to have broadened and has been used by different researchers to describe different situations. Generally, the term now seems to be used to describe any cell with the potential to differentiate towards a haemopoietic and endothelial lineage even if this potential is unbalanced, as described in the sections above discussing both haemogenic endothelium and the adult haemangioblast. The original haemangioblast theory, describing differentiation of a single cell to form both endothelial and haemopoietic daughter cells, has never been observed *in vivo*. A possible reason for this may be that *in vivo* a cell is unlikely to be in a situation where environmental cues would provoke such a simultaneous differentiation.

The second recent evolution in modern haemangioblast theory is that the haemangioblast appears to have a greater capacity for differentiation than previously

thought, encompassing smooth muscle differentiation *in vitro* (potentially via transdifferentiation of endothelial cells) (Ema and Rossant, 2003) and possibly sharing a common precursor with cardiac endothelium and muscle (Kattman et al., 2006).

The available data concerning the kinetics, characterisation, definition and even existence of the haemangioblast *in vivo* are currently open to interpretation. Whilst it is clear that the original simple description of the haemangioblast model illustrated in Figure 1-9 is unlikely to exist, a clear picture is yet to emerge. What is known is that haemopoietic and endothelial cells share a very close developmental and functional relationship. Further research *in vitro* and *in vivo* into signalling pathways important in the expansion, migration and commitment of these lineages will help further clarify the haemangioblast hypothesis.

1.3 Cell signalling in haemopoietic and vascular development

Little is known about the molecular signalling processes which regulate the formation, migration and differentiation of the haemangioblast. However, the use of *in vitro* knockout approaches has enabled the detailed study of the importance of key signalling molecules, the most prominent of which are discussed below. Further research in this area would contribute to a greater understanding of the development of the haemopoietic and endothelial lineages and their interactions. Important intrinsic (of embryonic origin) and extrinsic (of extra-embryonic origin) signalling pathways in haemopoietic and vascular development are summarised in Figure 1-12.

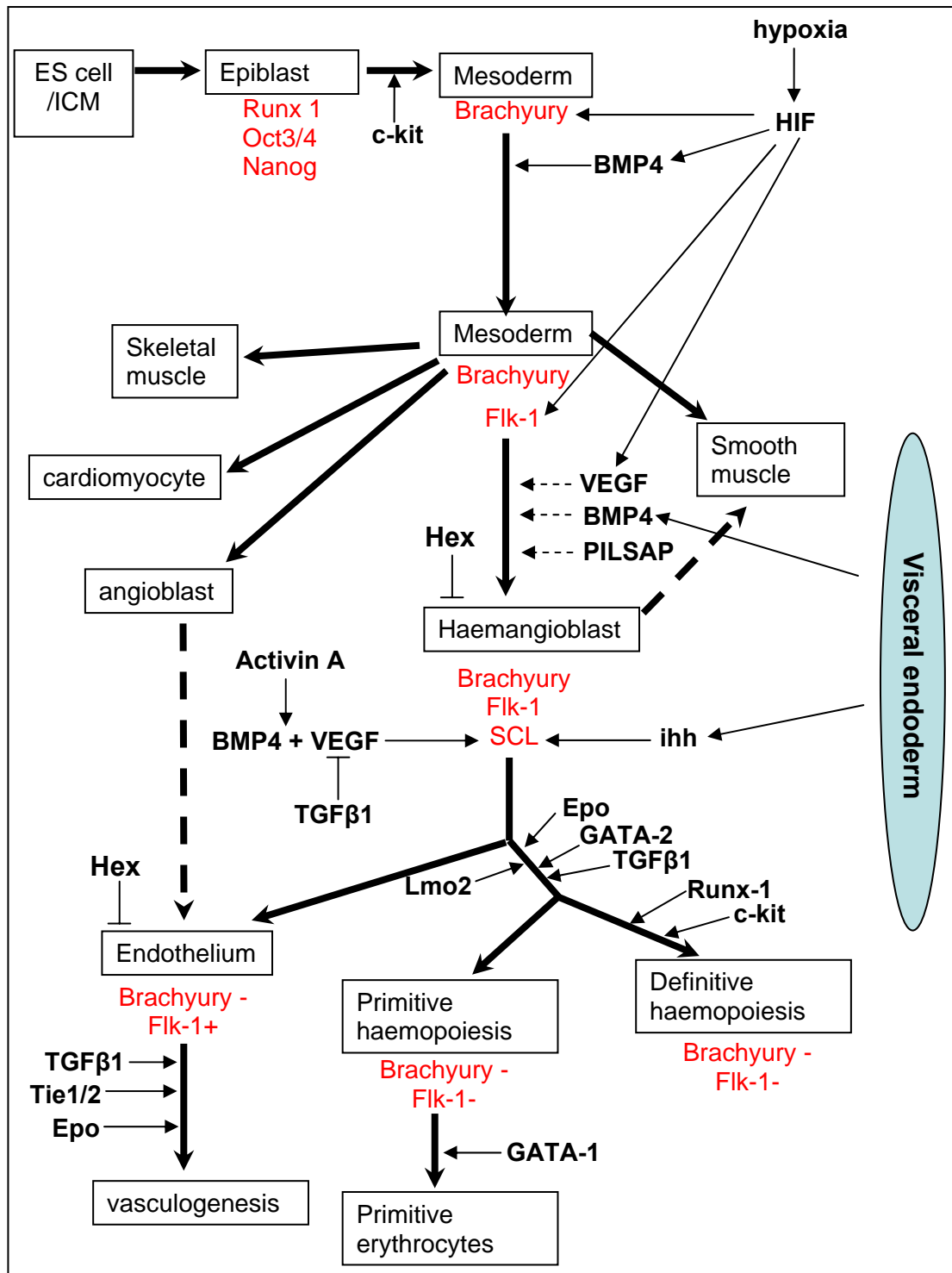


Figure 1-12 Important signalling pathways in haemangioblast commitment and differentiation. Diagram illustrating signalling molecules and transcription factor required in the development of the haemopoietic and endothelial lineages. Red wording indicates expression status of the molecule by cells of the lineage indicated. For further clarification and references see 1.3.1.1-1.3.1.5.

1.3.1 Signalling intrinsic to the embryo

1.3.1.1 *Brachyury*

Brachyury is a T box gene important in the regulation of mesodermal fate during gastrulation and is first detected in the primitive streak at E7 remaining until E9.5 (Kispert and Herrmann, 1994; Wilkinson et al., 1990). *Brachyury* null ES cells, reintroduced into the embryo, are unable to migrate, and remain in the primitive streak (Wilson et al., 1995). Knockout and lineage tracing experiments have demonstrated a further requirement for *Brachyury*, when coexpressed with Flk-1, in the formation of mesoderm fated to specify the haemangioblast (Fehling et al., 2003; Huber et al., 2004).

1.3.1.2 *Flk-1*

Flk-1 (foetal liver kinase-1) is one of the most studied signalling molecules with reference to the haemangioblast. Studies into the effect of Flk-1 gene knockout, published in 1995, provided supporting evidence for the haemangioblast theory, since mice null for this receptor tyrosine kinase died by E9.5 due to a failure in the formation of both haemopoietic and vascular systems (Shalaby et al., 1995). This early embryonic lethality led to the use of embryoid bodies to further investigate the role of Flk-1 and its ligand vascular endothelial growth factor (VEGF). *In vitro* studies support a role for Flk-1 in the migration of the putative haemangioblast, as well as its specification and differentiation (Choi et al., 1998; Hidaka et al., 1999; Kabrun et al., 1997; Schuh et al., 1999). The role of Flk-1 signalling in the haemangioblast is discussed further in section 1.4.1.1, Flk-1 signalling is discussed in section 1.4.2.

1.3.1.3 *Vascular Endothelial Growth Factor (VEGF)*

VEGF (VEGF-A), also known as vascular permeability factor (VPF), is a member of the platelet derived growth factor (PDGF) supergene family. Other vascular endothelial growth factors include VEGF-B, VEGF-C, VEGF-D and VEGF-E. VEGF-C and VEGF-D bind to VEGFR-3 (*fms*-like tyrosine kinase-4 (Flt-4)) that is expressed on lymphatic endothelial cells. VEGF-A and VEGF-B bind to VEGFR-1 (*fms*-like tyrosine kinase-1 (Flt-1)), a sister tyrosine kinase to Flk-1/KDR/VEGFR-2 that is also involved in the development of the embryonic vasculature. Flk-1 binds

VEGF-A, VEGF-C, VEGF-D and VEGF-E. VEGF-A is usually referred to simply as VEGF and is encoded by an eight exon gene on the short arm of chromosome 6. Alternative splicing of VEGF-A mRNA in humans gives rise to five different isoforms of the gene. The most abundant and biologically active of which, VEGF₁₆₅, forms a 46kDa disulphide linked homodimer (Zachary, 2001).

Mice with a heterozygous knockout of VEGF die at E10.5, seemingly due to delayed endothelial development, indicating either that VEGF action is dose-dependent or that there is a critical VEGF threshold required for endothelial development (Carmeliet et al., 1996; Ferrara et al., 1996). This view is supported by *in vitro* observations in which VEGF supports a dose-dependent differentiation of endothelial cells from embryonic stem cell-derived mesodermal cells (Hirashima et al., 2003). The VEGFR-1 knockout is also embryonic lethal, due to a severe disorganisation of the vasculature (Fong et al., 1995). However, a truncated form of VEGFR-1, lacking the tyrosine kinase domain, can rescue the knockout phenotype suggesting that its role is not in mediating signalling but rather in sequestering VEGF, for which it has a 10-fold higher affinity than Flk-1 (Waltenberger et al., 1994), in order to negatively modulate Flk-1 signalling (Hiratsuka et al., 1998). The transmembrane domain of VEGFR-1 appears to be important in this role allowing direct regulation of VEGF levels at the cell surface (Hiratsuka et al., 2005b). This negative modulatory role of VEGFR-1 is further supported by partial rescue of the VEGFR-1 knockout phenotype by addition of soluble VEGFR-1 (Kearney et al., 2004), or partial inhibition of Flk-1 (Roberts et al., 2004).

VEGF also binds to Neuropilins 1 and 2 which also play a role in vasculogenesis. Neuropilin-1 binds VEGF-A₁₆₅, VEGF-B, VEGF-E and the related placental growth factor-2 (PlGF-2). The Neuropilin-1 knockout phenotype includes impaired yolk sac vascularisation (Klagsbrun et al., 2002) and when Neuropilins-1 and 2 are knocked out together death occurs at E8.5 due to a severe failure in vascularisation (Takashima et al., 2002). When co-expressed with Flk-1, neuropilin-1 increased VEGF binding to Flk-1 and associated mitogenic effects (Soker et al., 1998). Modelling suggests this effect may be explained by binding of VEGF-A₁₆₅ to both neuropilin-1 and Flk-1 causing formation of a heterodimer (Mac Gabhann and Popel, 2005). The cytoplasmic domain of both neuropilins is small, indicating that

signalling is not transduced through them but rather that they act as co-receptors. The mechanism of their action is not yet fully elucidated. Other co-receptors include the Heparin sulphate proteoglycans which also enhance VEGF-A₁₆₅ mediated Flk-1 signalling (Ashikari-Hada et al., 2005).

1.3.1.4 Stem Cell Leukaemia factor

Scl or Tal1 is a basic helix-loop-helix transcription factor first identified in T cell acute lymphoblastic leukaemia. The Scl knockout mouse, like the Flk-1 knockout mouse dies between E9 and E10.5 due to anaemia (Shivdasani et al., 1995). In the absence of Scl haemopoiesis is undetectable, suggesting an early role in the specification of the primitive haemopoietic system (see Figure 1-12) (Porcher et al., 1996). Selective rescue of the Scl knockout in which the GATA-1 promotor was used to target Scl expression to the haemopoietic system, revealed a later, endothelial defect in the remodelling of the primitive vascular plexus to form a complex capillary system (Visvader et al., 1998). Many workers initially placed Scl as important in the formation of the haemangioblast, as in its absence blast colonies, indicative of formation of the Blast colony forming cell (BL-CFC), the *in vitro* equivalent of the haemangioblast, failed to form (Faloon et al., 2000; Robertson et al., 2000). However, further work demonstrated that cells from Scl null embryoid bodies, dissociated during the temporal window in which BL-CFC in wild-type embryoid bodies would be expected, were able to form secondary blast colonies, when a viral *Scl* vector rescue strategy was employed upon plating of cells into the secondary blast assay. Furthermore, genetic analysis found no *Scl* gene expression until day 1 of blast colony formation (D'Souza et al., 2005). This suggests that the BL-CFC can form in Scl null embryoid bodies but is unable to expand.

1.3.1.5 Other signalling factors

The complexity of signalling surrounding early patterning of the haemopoietic and vascular systems in the embryo is becoming ever more apparent with many recent publications revealing the involvement of novel molecules. Table 1-1 briefly summarises the signalling and transcription factors included in Figure 1-12.

Table 1-1 Signalling factors involved in haemopoietic and endothelial development

Signalling molecules	Role in haemopoietic and/or vascular endothelial development	References
Hex	Hex is a homeobox gene which appears to negatively regulate the haemangioblast and endothelial lineages	(Kubo et al., 2005)
Endoglin (CD105) and TGF β	Endoglin is a TGF β receptor. Expression is comparable to that of Flk-1 and the knockout is embryonic lethal due to vascular and cardiac defects. The TGF β knockout is embryonic lethal in 50% of animals due to defective yolk sac haemopoiesis and vasculogenesis. Defective endothelial differentiation results in weak vessels with reduced cellular adhesion. Park <i>et al.</i> reported inhibition of VEGF induced vascular and haemopoietic expansion by TGF β .	(Dickson et al., 1995; Li et al., 1999; Park et al., 2004; Perlingeiro, 2004)
puromycin insensitive leucine specific amino peptidase (PILSAP) also known as Arts1	A leucine and methionine specific metalloproteinase upregulated during endothelial differentiation. Inhibition of PILSAP using a dominant negative approach or siRNA inhibited growth and differentiation of haemopoietic, endothelial and vascular smooth muscle lineages.	(Abe and Sato, 2006)
Runt-related Transcription Factor-1 (Runx-1)	The mouse equivalent of AML-1, Runx-1 is a transcription factor of the core binding factor family. Runx-1 is required for definitive haemopoiesis and appears to play an earlier role negatively regulating mesodermal commitment towards to haemangioblast	(Lacaud et al., 2002; Lacaud et al., 2004)
Erythropoietin (Epo)	Epo is a cytokine essential for erythropoiesis but may also be involved to endothelial development. Epo treatment enhanced migration, endothelial marker expression and tube formation and decreased apoptosis in embryoid bodies, embryoid body derived endothelial cells and human endothelial progenitor cells.	(Muller-Ehmsen et al., 2006)
Thrombopoietin (TPO) and its receptor c-mpl	A primary physiological regulator of platelet production which also plays a role in haemopoietic stem cell (HSC) function. TPO treatment or conditional activation of its receptor c-mpl has been shown to augment BL-CFC formation <i>in vitro</i>	(Perlingeiro et al., 2003)
GATA binding protein-1 and -2	GATA-1 and 2 are zinc finger transcription factors. GATA-1 null embryos are unable to proceed beyond the proerythroblast stage of erythropoiesis though early haemopoiesis proceeds normally possibly reflecting redundancy with GATA-2. GATA-2 is embryonic lethal due to failure of primitive haemopoiesis. <i>In vitro</i> studies show upregulation of GATA-2 on day 2 in blast colonies, consistent with the timing of haemopoietic commitment	(D'Souza et al., 2005; Fujiwara et al., 1996; Tsai et al., 1994)
Tyrosine kinase with Ig and EGF factor homology domains (Tie)-1 and 2	Belong to a distinct family of receptor tyrosine kinases expressed mainly in endothelial cells. Tie-1 is necessary for the establishment of vessel structural integrity whereas Tie-2 is important for vascular network formation. In addition Tie-2 has been shown to be involved in adult but not fetal definitive haemopoiesis.	(Puri and Bernstein, 2003; Sato et al., 1995; Takakura et al., 1998)

Lim Domain Only -2 (Lmo2)	Lmo2 null mice die at E10.5 due to failure of yolk sac haemopoiesis. Later work has shown an additional role in adult definitive haemopoiesis. The knockout phenotype is similar to that of SCL (see 1.3.1.3) Biochemical analysis and work in zebrafish suggest that it may function in a complex with SCL and GATA-1.	(Patterson et al., 2007; Wadman et al., 1997; Warren et al., 1994; Yamada et al., 1998)
c-kit	c-kit is a receptor tyrosine kinase required for embryonic and adult haemopoiesis. c-kit null ES cells die upon LIF withdrawal though c-kit null ICM and epiblast cells display a normal phenotype.	(Bashamboo et al., 2006; Ronnstrand, 2004; Russell, 1979)

1.3.2 Extrinsic factors

Additional to signalling intrinsic to the embryo are those signals of extrinsic origin. Whilst more difficult to model *in vitro*, extrinsic signalling has been proven to play an important role in haemangioblast development. The two chief sources of extrinsic signalling are the visceral endoderm and signalling as a result of hypoxia.

1.3.2.1 Hypoxia

Hypoxia plays a crucial role in the initial generation of mesoderm, and formation of vascular and haemopoietic derivatives in early development. Its effects are mediated through Hypoxia inducible transcription factors (Hif), Hif1 α (which in normoxic conditions is degraded but is stabilised in hypoxic conditions by inhibition of Hif1 α hydroxylase I) and Hif1 β which is constitutively expressed. The effects of hypoxia include accelerated expression of Bmp4, Flk-1 and *Brachyury*, all of which promote the early development of haemopoietic and vascular endothelial lineages (Ramirez-Bergeron et al., 2004). Hif1 β knockout mice have defects in vascular remodelling and haemopoiesis in the extraembryonic yolk sac (Maltepe et al., 1997) and Hif1 β ^{-/-} ES cells show reduced expression of Flk-1 and a decreased number of blast colonies in *in vitro* models (Ramirez-Bergeron et al., 2004). Vascular Endothelial Growth Factor (VEGF) plays an important role in the embryonic response to hypoxia. Binding of Hif1 α to the hypoxia response element (HRE) of VEGF in ES cells increases VEGF concentrations thus inhibiting apoptosis both *in vitro* (Brusselmans et al., 2005) and *in vivo* in quail eggs (Nanka et al., 2006).

1.3.2.2 Visceral endoderm

Explant culture experiments have demonstrated the importance of factors secreted from the visceral endoderm in specifying haemopoietic and vascular endothelial cell fate. Embryos from which the visceral endoderm is removed fail to express β -H1 - globin, Gata1 or Flk-1 but can be rescued by donor visceral endoderm from other embryos (Belaousoff et al., 1998). Candidates for these important soluble factors include Indian hedgehog (ihh) and Bmp4 (Dyer et al., 2001; Farrington et al., 1997). The relative importance of Bmp4 to haemopoietic and endothelial specification is difficult to quantify as it plays such an important role in earlier mesoderm patterning events. However, recent work by Park *et al.*, demonstrated that inactivation of Bmpr1a (Alk3), a receptor for Bmp4, resulted in embryonic lethality due to vascular but not haemopoietic defects (Park et al., 2006). Ihh is able to substitute for visceral endoderm and promote haemopoietic and endothelial development in epiblasts stripped of visceral endoderm. Whether this is physiological has not yet been established (Dyer et al., 2001). However, the ihh knockout mouse does display vascular defects and dies *in utero* suggesting ihh does have a physiological role to play in vasulogenesis if not the initial specification of haemopoietic and endothelial lineages which are both able to form in the absence of ihh (Byrd et al., 2002; Vokes et al., 2004).

1.4 The role of Flk-1 in early development

Flk-1, a receptor tyrosine kinase, is also known as vascular endothelial growth factor-receptor-2 (VEGFR-2), and Kinase Domain Receptor (KDR) in humans. It plays a key role in the formation of the circulatory system in the embryo where it is expressed on the putative haemangioblast and endothelial lineages (Schuh et al., 1999; Shalaby et al., 1995). Expression of the receptor is maintained on adult endothelial cells in which it mediates angiogenesis, making it an important target in the pathology of many human diseases including cancer, Rheumatoid Arthritis, ocular neovascularising disease and cardiovascular disease (Zachary, 2001).

1.4.1 The role of Flk-1 in embryogenesis and *in vitro* models of embryogenesis

1.4.1.1 *The role of Flk-1 in differentiation to haemopoietic and endothelial lineages*

Deletion of both Flk-1 alleles in the mouse is embryonic lethal between E8.5 and E9.5 due to a lack of developing vasculature. Knocking out one allele has no effect suggesting that the requirement for Flk-1 is not concentration dependent (Shalaby et al., 1995; Shalaby et al., 1997). The yolk sac blood islands are absent at E7.5 as mesenchymal aggregates fail to form and there are no organised blood vessels in Flk-1 null embryos. There are also very few haemopoietic progenitors, although it is not clear whether this is a direct effect of the Flk-1 knock-out or occurs due to the lack of blood vessels adversely influencing the haemopoietic microenvironment. Cells in the Flk-1null animals expressing lacZ under the Flk-1 promotor were found in the amnion and mesothelium, indicating that Flk-1 may normally be involved in their migration (Shalaby et al., 1997) .

The embryonic lethality of the Flk-1 knockout mouse makes it difficult to investigate its role fully *in vivo*. However, expression studies have shown that Flk-1 is expressed on early haemopoietic as well as endothelial progenitors in the yolk sac (E8.5-9.5) (Kabrun et al., 1997) and Flk-1 involvement has been demonstrated in the development of both lineages in embryoid body models (Hidaka et al., 1999; Kabrun et al., 1997; Nishikawa et al., 1998a). Fehling *et al.*, identified Flk-1 along with *Brachyury* as markers of the population of embryoid body cells from which the BL-CFC arose (Fehling et al., 2003) and more recent experiments by Huber *et al.*, identified cells in the primitive streak which again expressed both Flk-1 and *Brachyury* and which were able to clonally differentiate *in vitro* into both haemopoietic and endothelial lineages (Huber et al., 2004) (see section 1.2.4.3).

The precise role of Flk-1, particularly in haemopoietic development, has been difficult to elucidate. Reported expression of Flk-1 on early haemopoietic progenitors is lost in most definitively differentiated haemopoietic cells (Kabrun et al., 1997; Nishikawa et al., 1998a) though it is reportedly expressed on (some) haemopoietic

stem cells in the adult (Gerber et al., 2002; Ziegler et al., 1999). Initially it was thought that down-regulation of Flk-1 in haemopoietic cells and its retention in endothelial cells may be an important signal in the differentiation of haemangioblast cells (Yamaguchi et al., 1993). Subsequent research demonstrated that haemopoietic differentiation *was* possible in the absence of Flk-1 though to a lesser extent and with greater dependency on the correct microenvironment suggesting a role in cell migration and proliferation (Hidaka et al., 1999; Schuh et al., 1999). A migratory role is supported by the presence of Flk-1 positive haemopoietic progenitors at E7.5 in the embryo despite an absence of such progenitors at E8.5 (Schuh et al., 1999; Shalaby et al., 1995; Shalaby et al., 1997) and by the apparent importance of VEGF-A, a ligand for Flk-1, in the migration of VEGFR positive cells from the posterior to the anterior of the embryo (Hiratsuka et al., 2005a).

One important question is whether Flk-1 is required for haemopoietic differentiation? The answer may be sometimes. *In vitro* work has reported haemopoietic progenitors to both express Flk-1 and be derived from a Flk-1 positive population (Kabrun et al., 1997; Nishikawa et al., 1998a) although Flk-1 knockout ES cells are still able to form cells of the haemopoietic lineage *in vitro* (Hidaka et al., 1999). Work published by Ema *et al.*, demonstrated that Flk-1 expression is not always indicative of function as its early expression is both promiscuous and extensive within the mesodermal lineages which may explain this apparent paradox (Ema et al., 2006). Additionally, new research using a Flk-1 concentration-responsive cre-lox P-driven fluorescent reporter system suggests that the origins of haemopoiesis *in vivo* are heterogenous with haemopoietic cells arising from both Flk-1 positive (possibly the haemangioblast population) and Flk-1 negative (possibly representing a more lineage restricted population – see 1.2.3.6) population (Ueno and Weissman, 2006). It is possible that Flk-1 is required for a subset of haemopoiesis, perhaps that arising from the bipotential haemangioblast but not for all early haemopoiesis.

The role of Flk-1 in the formation of the vascular endothelial lineage appears more straightforward. The Flk-1 knockout animal forms no blood vessels (Shalaby et al., 1995), Flk-1 is required for adult angiogenesis and its expression remains on adult endothelial cells. The Flk-1 concentration-responsive cre-lox P-driven fluorescent reporter system used by Ueno and Weissman demonstrated that all endothelial cells

in the yolk sac were derived from Flk-1 positive precursors (Ueno and Weissman, 2006). *In vitro*, however, there are reports of endothelial formation from Flk-1 knockout ES cells (Schuh et al., 1999). Though the identification of the endothelial cells was made through using expression of endothelial markers (some of which have since been demonstrated to be expressed on the haemopoietic lineage; (Fleming, 2005) the panel of markers used still seems to define the endothelial cell. Perhaps this is once again evidence for a migratory role for Flk-1 which can be overcome by a differing microenvironment *in vitro* or alternatively evidence of adaptation of the Flk-1 knockout ES cells. More recent research, in Zebrafish, has postulated a role for Flk-1 signalling in the specification of arterial and venous fates. Disruption of the *gridlock* gene in Zebrafish led to formation of insufficient numbers of arterial cells and reduction or loss of the aorta. This phenotype could be suppressed by injection of VEGF cDNA or addition of a small molecule GS4012, postulated to activate VEGF signalling, suggesting a role for Flk-1 in arterial specification (Hong et al., 2006).

1.4.1.2 The role of Flk-1 in cardiac and neural development

In addition to its role in haemopoietic and endothelial development Flk-1 has also been implicated in both cardiac and neural development. The first indication that Flk-1 was involved in developmental processes other than haemopoietic and endothelial development came with the identification of its expression in retinal progenitor cells (Yang and Cepko, 1996). Flk-1 activation of the mitogen activated protein kinase (MAPK) pathway has subsequently been demonstrated as a mitogenic signal in cultured peripheral ganglia and central nervous system neurons (Khaibullina et al., 2004; Rosenstein et al., 2003; Sondell et al., 1999) and Flk-1 has also been implicated in neural protection and development (reviewed Rosenstein and Krum, 2004).

The involvement of Flk-1 in cardiac development has been discovered more recently. The heart is formed by two populations of mesodermal cells which migrate through the primitive streak, the anterior splanchnic mesoderm which contributes to the initial stages of cardiogenesis and the pharyngeal mesoderm which migrates to the site of cardiogenesis later at around E9.5 (Kelly, 2005). The role of Flk-1 in the initial stages of cardiogenesis is illustrated in Figure 1-13.

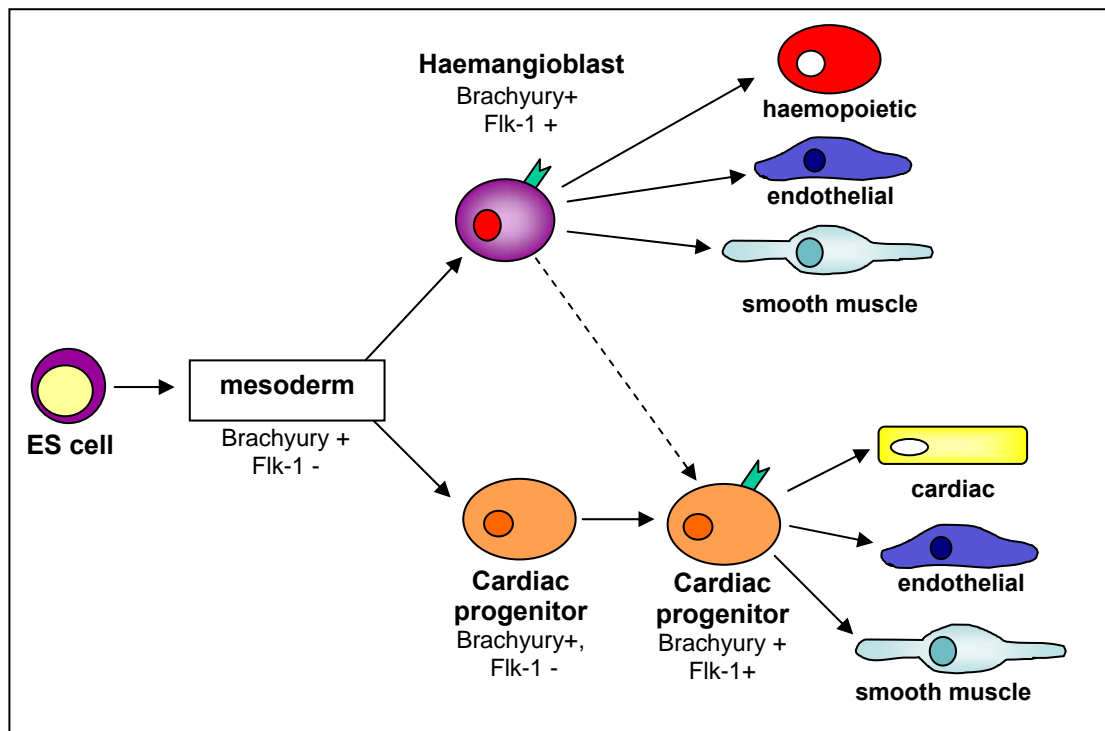


Figure 1-13. Proposed role of Flk-1 in specifying mesodermal differentiation towards either a cardiac or haemangioblastic fate. Cells with haemangioblastic or cardiac potential can be separated at day 3.25 of embryoid body development based upon their expression of Flk-1. Cells with haemangiogenic potential express Flk-1 at day 3.25 whereas cells with cardiac potential don't upregulate expression until day 4.25 (Kattman et al., 2006).

Lineage tracing experiments identified a Flk-1 positive intermediate population which appears to develop as cells exit the primitive streak and begin to migrate towards the cardiac crescent (Ema et al., 2006). Further study, using embryoid bodies as a model of cardiogenesis (Boheler et al., 2002; Sachinidis et al., 2002), demonstrated an increase in expression of cardiac markers in response to VEGF and a marked decrease upon treatment with an anti-Flk-1 antibody (Chen et al., 2006). Work by the Keller group first identified that the cardiogenic mesoderm population was derived from a population which was *Brachyury* positive but Flk-1 negative at E3.25, the point of BL-CFC/haemangioblast formation (Kouskoff et al., 2005) and then demonstrated that at E4.25 a new Flk-1 positive population was generated which was identified to contain the majority of cardiac potential within the differentiation model and which had the potential to form cardiac, endothelial and visceral smooth muscle lineages (Kattman et al., 2006).

1.4.2 Flk-1 signalling

The signalling mechanisms mediated by the receptor tyrosine kinase Flk-1 have been little characterised in the embryo, but due to its importance therapeutically, research has been conducted in primary human endothelial cells and cell lines on the human equivalent of murine Flk-1, KDR. Flk-1/KDR has seven immunoglobulin-like domains extracellularly and a split tyrosine kinase domain intracellularly (see Figure 1-14). It binds its dimeric ligand, vascular endothelial growth factor (VEGF), in the second and third Ig-like domains which leads to receptor dimerisation and transphosphorylation of tyrosine residues in its intracellular tail. KDR activation initiates a series of signalling cascades, illustrated in Figure 1-15, which in the adult mediate cell migration, survival and proliferation (Neufeld et al., 1999).

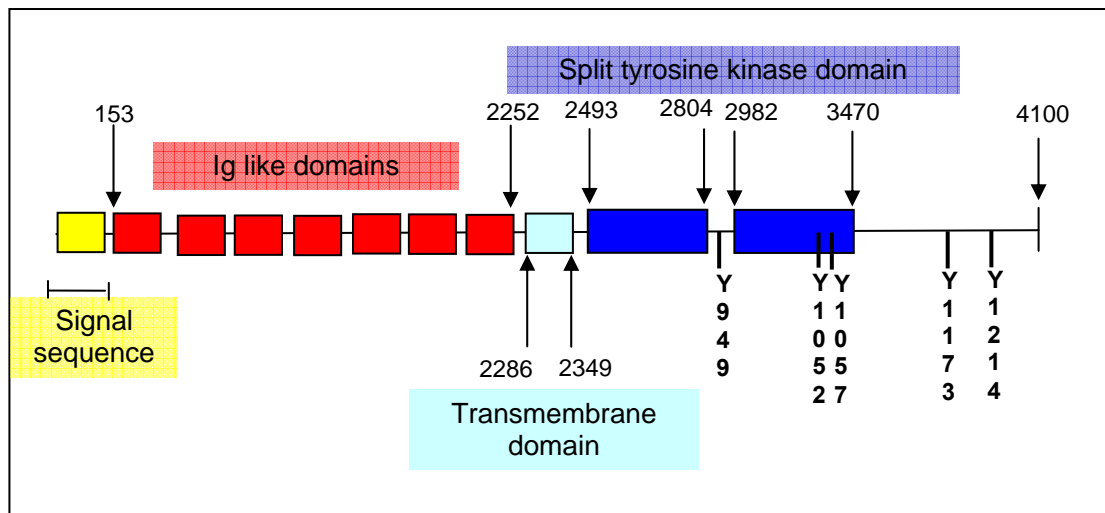


Figure 1-14 Schematic of the Flk-1 receptor based upon sequence information for NM_010612 from NCBI (<http://www.ncbi.nlm.gov>) accessed on November 11th 2006. Positions of tyrosine phosphorylation on the cytoplasmic portion of the receptor upon receptor stimulation are illustrated. Numbers illustrate the base pair position in the cDNA sequence from the start codon at 1 and (in bold) amino acid positions.

VEGF causes dimerisation of the KDR receptor and transphosphorylation of key tyrosine residues in the cytoplasmic tail including tyrosines at positions 951, 1054, 1059, 1175 and 1214 (Lamallice et al., 2004; Matsumoto et al., 2005; Takahashi et al., 2001). Phosphorylation of Y1054 and Y1059 (1052 and 1057 in murine Flk-1) increases intrinsic tyrosine kinase activity of Flk-1 (Dougher and Terman, 1999; Kendall et al., 1999). Proteins containing a Src homology-2 (SH2) domain (a module

of ~100 amino acids which binds the phospho-tyrosine via a conserved domain and binds 3-6 residues C-terminal to the tyrosine via a variable pocket which confers specificity) are then able to bind to phosphorylated tyrosine residues to potentiate Flk-1 signalling (Guo et al., 1995). The following major signalling pathways activated through the receptor are illustrated in Figure 1-15; phospholipase C- γ (PLC- γ) related signalling (McLaughlin and De Vries, 2001), the PI3K pathway and the Mitogen Activated Protein Kinase (MAPK) pathway (discussed further in sections 1.4.2.1, 1.4.2.3 and 1.4.2.2 respectively). SHP-1 (SH2 domain containing protein tyrosine phosphatase) (Guo et al., 2000; Kroll and Waltenberger, 1997) and SHP-2 (Gallicchio et al., 2005; Kroll and Waltenberger, 1997) are also thought to associate with the Flk-1 receptor and are possibly involved in attenuating KDR signalling.

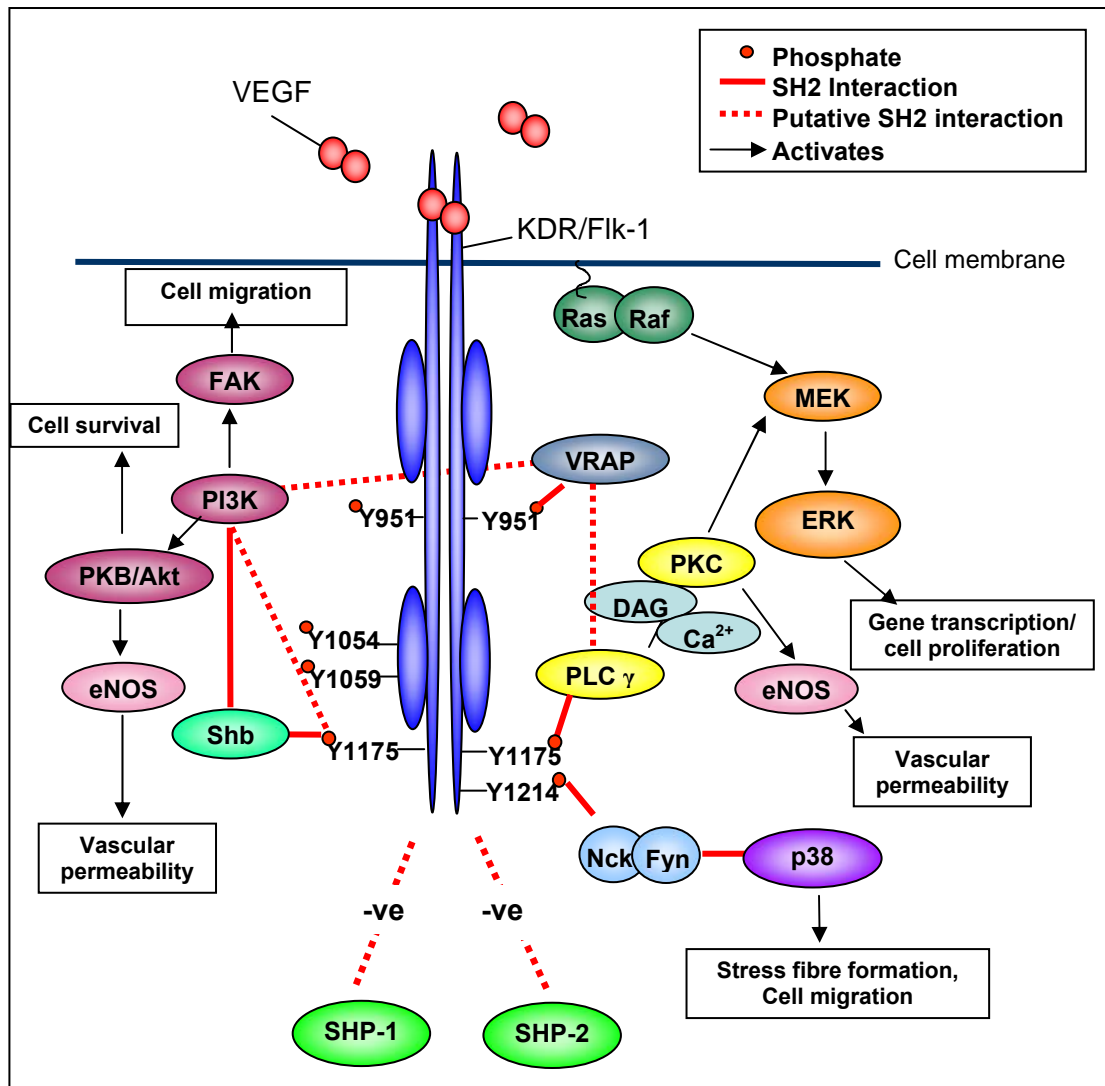


Figure 1-15 Summary of KDR signalling in human endothelial cells. Modified from (Olsson et al., 2006). Binding of the VEGF dimer causes receptor dimerisation of phosphorylation of the key tyrosine residues illustrated. Signalling is then potentiated by the binding and phosphorylation of SH2 domain containing intermediate signalling molecules. Activation of the signalling cascades illustrated leads to a variety of effects on endothelial cell proliferation, survival, migration, vascular permeability and gene transcription. Signalling is thought to be negatively regulated by dephosphorylation of tyrosine residues by SHP-1 and SHP-2 tyrosine phosphatases though the specific residues involved in this have not yet been elucidated. VEGF – vascular endothelial growth factor; KDR – kinase domain receptor, human homologue of Flk-1; FAK-focal adhesion kinase; PI3K-phosphoinositide 3-kinase; PKB-protein kinase B; eNOS-endothelial nitric oxide synthase; Shb-src homology 2 domain-containing transforming protein B; SHP -Src homology domain containing protein tyrosine phosphatase; Nck-non-catalytic region of tyrosine kinase adaptor protein; PLC-phospholipase C; DAG-diacyl glycerol; PKC-protein kinase C; VRAP-VEGF-receptor associated protein; MEK-MAP-ERK kinase; ERK1/2-extracellular-related kinase-1 and 2

1.4.2.1 Phospholipase C

Phospholipase C- γ is an SH2-containing protein activated upon phosphorylation by a receptor tyrosine kinase. Activated PLC- γ cleaves the membrane lipid phosphoinositolbisphosphate (PI(3,4)P₂) to form inositol triphosphate (IP₃) and diacylglycerol (DAG) which is required for activation of the novel protein kinase C (PKC) isoforms. IP₃ induces calcium release from the endoplasmic reticulum, allowing activation of the classical PKC isoforms which are dependent upon both DAG and calcium for activation (Carpenter and Ji, 1999). Activation of PKCs leads to activation of downstream signalling pathways including MAPK signalling (Doanes et al., 1999; Glikli et al., 2001) and activation of eNOS (endothelial Nitric Oxide Synthase) (Lin and Sessa, 2006) which mediate proliferation and cellular permeability respectively.

Activation of PLC- γ by KDR is thought to occur either via a direct SH2 interaction with between the lipase and the receptor, via an adaptor molecule or using both mechanisms. The main candidate tyrosine for a direct PLC- γ interaction is Y1175 initially identified using adenovirally introduced point-mutated KDR into the NIH-3T3 fibroblast cell line (Takahashi et al., 2001). Y951 has also been identified as a potential candidate for PLC- γ activation mediated via Y951 binding by the adaptor protein VRAP (VEGF Receptor Associated Protein, also known as TSad and Sh2d2a) (Matsumoto et al., 2005; Wu et al., 2000b). The identity of the PKC isoform activated downstream of PLC- γ has not been confirmed. Candidates include PKC- δ (Glikli et al., 2001; Kuriyama et al., 2004) PKC- α , and PKC- μ (PKD) downstream of PKC- α (Wong and Jin, 2005).

1.4.2.2 Mitogen Activated Protein Kinase Signalling

Mitogen Activated Protein Kinase signalling is transduced through 4 main pathways which respond to a combination of growth factors, mitogens and stress and transduce their signals through a phosphorylation cascade. The pathways of particular relevance in endothelial cells appear to be the VEGF activated Extracellular Related Kinase (ERK1/2) pathway (Boulton et al., 1991) and the VEGF and stress activated p38 MAPK pathway (Rouse et al., 1994) both of which are illustrated in Figure 1-16.

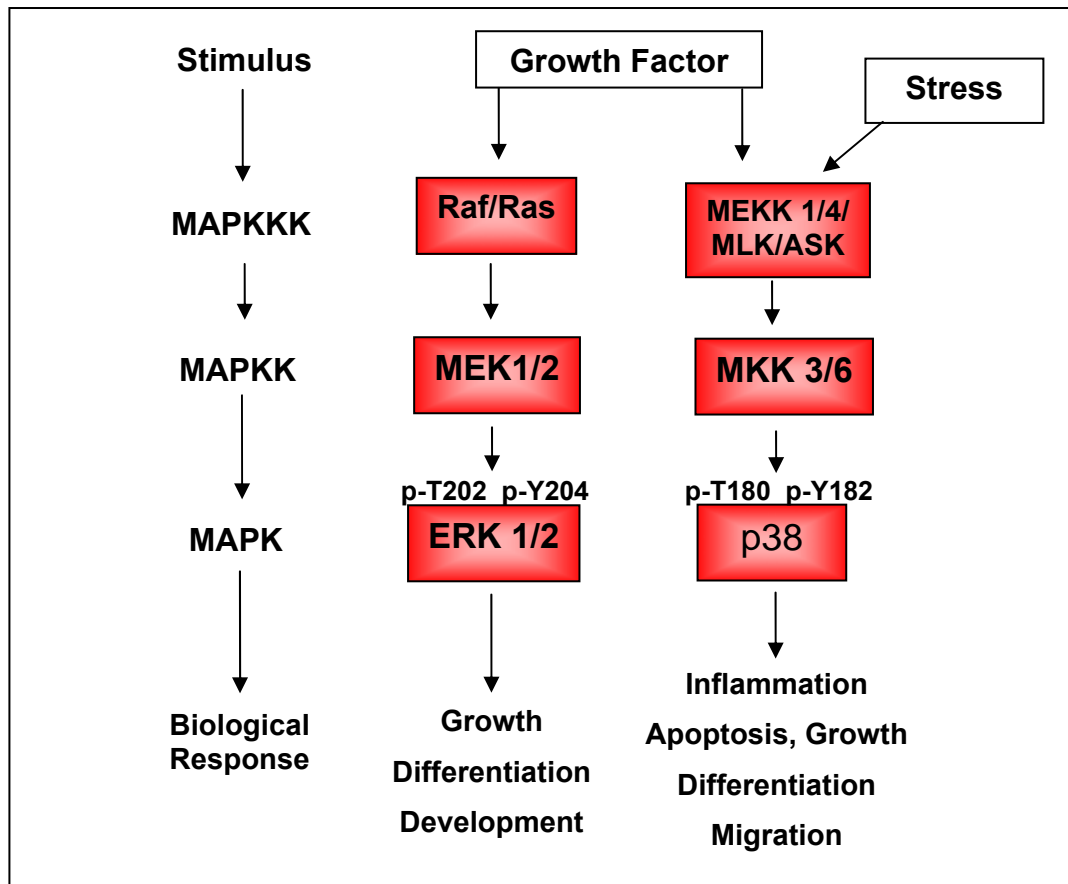


Figure 1-16 Illustration of the MAPK pathways activated by Flk-1 signalling in endothelial cells. MAP kinase signalling is organised in a three-tier hierarchy. Stimulation of a growth factor receptor (or in the case of p38 activation, stress) causes phosphorylation and activation of MAP kinase kinase kinase which in turn phosphorylates MAP kinase kinase activating it and enabling phosphorylation of the MAP kinase which mediates the biological response.

1.4.2.2.1 ERK1/2 signalling

Receptor tyrosine kinases typically activate the MAPK pathway through Grb2-Sos-Ras-Raf. In the activated KDR receptor, however, the MAPK signalling pathway is reportedly activated through Protein kinase C (PKC), which is itself activated by PLC- γ (Doanes et al., 1999; Gliki et al., 2001). KDR activation does activate Ras, possibly through the Shc-Grb-2 complex, but expression of a dominant negative Ras mutant does not inhibit MAPK activation despite inhibiting Ras mediated MAPK activation by other receptor systems in human umbilical vein endothelial cells (HUVECs) (Doanes et al., 1999). Two structurally unrelated inhibitors of PKC (Calphostin and Bisindoylmaleimide) can block MAPK activity (Doanes et al., 1999; Gliki et al., 2001) though Bisindoylmaleimide is not selective at the concentration

used (5 μ M) and may have inhibited GSK-3 α or β or Protein Kinase-A (PKA). Despite the apparent reliance of MAPK activation on PKC, a truncated Raf, lacking its kinase domain, also abrogated MAPK signalling in response to activation of KDR, indicating the Ras-Raf interaction still has a role to play in MAPK signalling through KDR (Doanes et al., 1999). In addition a study by Yashima *et al.* demonstrated variation in the mechanism of MAPK activation between human cell sources. VEGF appeared to activate the MAPK pathway through a PKC-mediated mechanism in HUVECs and human aortic endothelial cells (HAECs), but in human subcutaneous microvascular endothelial cells (HMVECs) MAPK activation seemed dependent only on the classical Ras-Raf pathway (Yashima et al., 2001). A PKC δ inhibitor (rottlerin) inhibits MAPK signalling whereas Go6976, which inhibits PKC α , does not, indicating MAPK is at least partially activated by the delta form of PKC (Gliki et al., 2001). Although this is the first example of a receptor tyrosine kinase activating the MAPK kinase pathway through PKC, accompanied by a Ras-Raf interaction, it does occur through other receptors including seven transmembrane G α q G protein-coupled receptors (vanBiesen et al., 1996).

1.4.2.2.2 p38 signalling

KDR can activate the p38 MAPK pathway in endothelial cells leading to increased stress fibre formation and promoting migration (Yashima et al., 2001). Recently Lamalice *et al.*, demonstrated that association of the adaptor proteins Nck and Fyn with phosphorylated Y1214 in KDR mediated activation of this pathway (Lamalice et al., 2006).

1.4.2.3 Phosphoinositide-3-kinase signalling

Phosphoinositides are a family of integral membrane lipids which can act as second messengers in cell signalling when phosphorylated at specific residues on their inositol rings. The phosphoinositide-3-kinases (PI3K) are a family of kinases which phosphorylate the 3' position of the inositol ring. Phosphorylation of inositol lipids at this position allows binding and membrane localisation of proteins which contain a pleckstrin homology (PH) domain such as Protein Kinase B (PKB) (Cantley, 2002; Vanhaesebroeck et al., 2001; Vanhaesebroeck and Waterfield, 1999). PI3K signalling is negatively regulated by the PTEN (Phosphatase and Tensin homolog) phosphatase. Mutation causing loss of PTEN activity causes constitutive activation

of the PI3K pathway leading to increased cell proliferation and decreased apoptosis. As PTEN is the most commonly lost tumour suppressor in human cancers, the PI3K pathway is of great interest as a drug target (Hennessey et al., 2005; Simpson and Parsons, 2001).

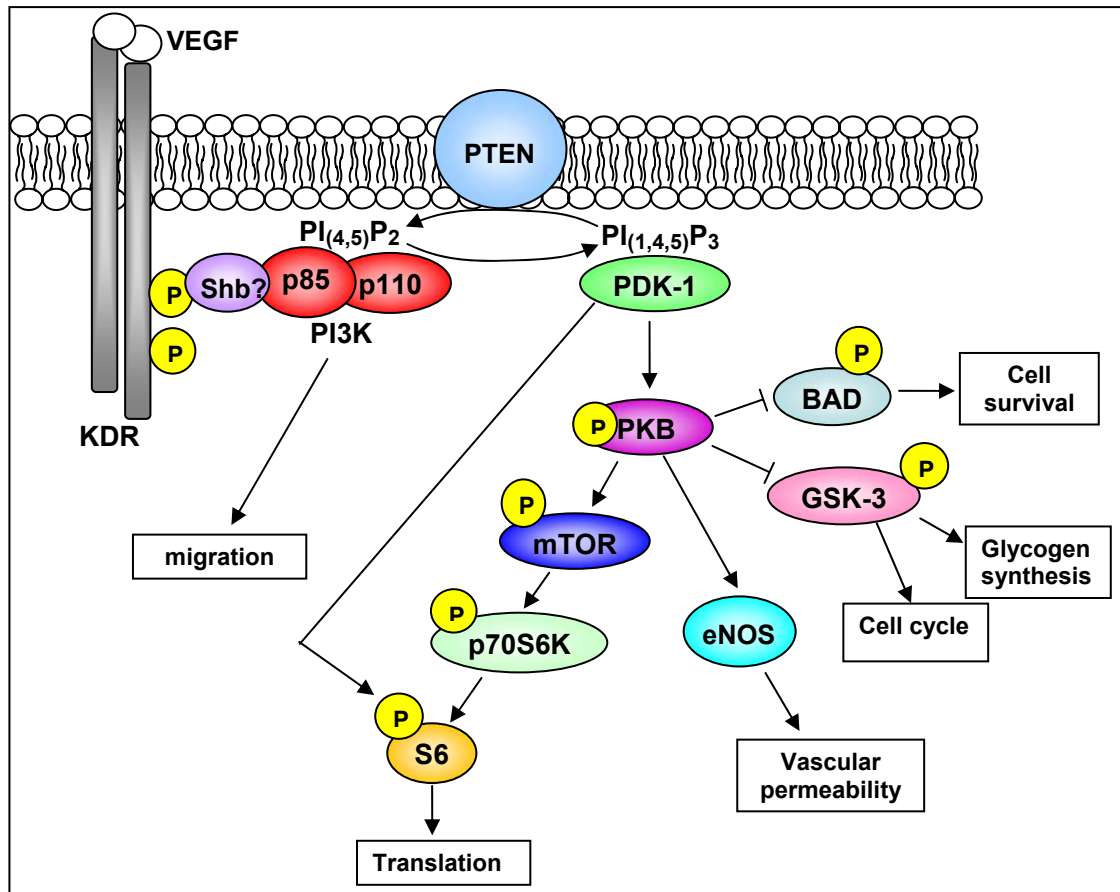


Figure 1-17 Schematic of PI3K (class IA) signalling mediated by KDR in human adult endothelial cells. Ligand binding leads to activation of PI3K by an as yet unconfirmed mechanism possibly involving Shb. The most common PI3K product is PI(3,4,5)P₃ which binds to the PH (pleckstrin homology) domain of PDK-1 localising it to the membrane where it phosphorylates and activates PKB/Akt. This initiates a phosphorylation cascade leading to the phosphorylation of downstream substrates including BAD, GSK-3, eNOS and mTOR. The mechanism of KDR-mediated PI3K involvement in migration is unresolved. PTEN-Phosphatase and tensin homologue; Shb-Src homology 2 domain-containing transforming protein B; PI3K-phosphoinositide 3-kinase; p85-class IA PI3K regulatory subunit; p110-class IA PI3K catalytic subunit; PDK-1-Phosphoinositide-dependent kinase-1; PKB-Protein kinase B; BAD-BCL-associated death promoter; GSK-3-Glycogen synthase kinase 3; mTOR-Mammalian target of rapamycin; eNOS-Endothelial nitric oxide synthase; p70S6K-p70S6 kinase; S6-S6 ribosomal protein.

PI3K signalling mediated through KDR activation (illustrated in Figure 1-17), has been demonstrated to play a role in cell migration via focal adhesions (Holmqvist et al., 2004), cell survival via Akt/PKB (Fujio and Walsh, 1999) and vascular permeability via eNOS activation (Bates and Harper, 2002; Fulton et al., 1999; Lin and Sessa, 2006).

There are 3 classes of PI3Ks, characterised according to their substrate specificity and structural characteristics;

Class I PI3K are heterodimeric, composed of a regulatory and a catalytic subunit. They preferentially phosphorylate phosphatidylinositol-4,5 bis-phosphate (PtdIns(4,5)P₂) (Vanhaesebroeck and Waterfield, 1999). The class is further divided into Class IA and Class IB. Class IA signal downstream of tyrosine kinase receptors and are recruited to the receptor via the p55 or p85 SH2 domain-containing regulatory subunits. Catalytic activity is mediated by p110 α , β or δ catalytic subunits (Cantley, 2002; Cantrell, 2001; Vanhaesebroeck et al., 2001; Vanhaesebroeck and Waterfield, 1999). Class IB PI3Ks signal downstream of G protein coupled receptors. They are recruited to the receptor via the p101 regulatory subunit and kinase activity is mediated the p110 γ catalytic subunit.

Class II PI3K isoforms (α , β and γ) are activated downstream of polypeptide growth factor receptors, chemokine receptors and integrins. They are characterised by their C-terminal C2-domain which facilitates calcium-independent phospholipid binding and a phox homology (PX) domain which binds to PtdIns(3)P and PtdIns(3,4)P₂ tethering the classII PI3K to the membrane (Cantrell, 2001).

Class III PI3K exist in a complex with a serine/threonine kinase. It phosphorylates only PtdIns (Cantrell, 2001).

Research into the role of PI3Ks downstream of KDR/Flk-1 has focussed on the class IA PI3Ks. Phosphorylation of Y801 and Y1175, in the cytoplasmic portion of Flk-1, has been demonstrated to be necessary for the activation of PI3K in a chimeric receptor construct. The construct contained the extracellular domain of human *c-fms*, fused with the transmembrane and intracellular domains of Flk-1, and was expressed

in porcine aortic endothelial cells (PAEs). Inhibition of the PI3K pathway in these cells with either wortmannin or rapamycin, or site directed mutagenesis of Y801 and Y1175, abrogates the chimeric receptors ability to stimulate cell growth implying that PI3K signalling downstream of Flk-1 is important for endothelial cell proliferation (Dayanir et al., 2001). More recently, work using the same PAE cell line but with a point mutation at Y1175 in the cytoplasmic tail of the chimeric receptor, demonstrated a decrease in Shb phosphorylation. Subsequent work in PAE cells expressing KDR demonstrated that decreasing Shb expression led to loss of stimulation of PI3K activity, in particular, PI3K-mediated migration. This suggests that a direct or indirect interaction between Y1175 in the cytoplasmic tail of the KDR receptor and Shb may potentiate PI3K signalling (Holmqvist et al., 2004). Like PLC- γ , the p85 subunit of PI3K is able to bind the adaptor protein VRAP which binds to Y951 in the cytoplasmic tail of Flk-1. An associated with the adaptor protein Gab-1 has also been reported (see section 1.4.2.4) suggesting that association with the adaptor proteins Shb and Gab-1 may be another route by which KDR mediates activation of PI3K (Matsumoto et al., 2005; Wu et al., 2000b).

1.4.2.4 Other Flk-1 mediated signalling

The activation of different signalling pathways by one receptor is often achieved through adaptor molecules. Gab-1 has recently been identified as one such important adaptor for KDR signalling. Following VEGF stimulation of Bovine Aortic Endothelial Cells (BAECs), Gab-1 phosphorylation increased and the protein co-immunoprecipitated with Grb-2, Shc, PI3K, SHP-2 and PLC- γ . Additionally, siRNA knockdown of Gab-1 in human microvascular endothelial cells (HMVECs) demonstrated a decrease in VEGF stimulated phosphorylation of PLC- γ , ERK1/2, Src and PKB/Akt indicating functional involvement of Gab-1 in mediating signalling downstream of KDR (Laramée et al., 2007).

Shear stress is an important mediator of vasculogenesis and angiogenesis. Yamamoto *et al.*, demonstrated an upregulation and activation of Flk-1 on embryonic stem cells subjected to shear stress (Yamamoto et al., 2005). Wang *et al.*, demonstrated that both VEGF and shear stress could activate NF κ B-mediated gene transcription via Akt/PKB-mediated activation of I κ B kinase (IKK) which in turn phosphorylates I κ B causing its degradation and the release and translocation to the nucleus of NF κ B. In

this case Akt/PKB activation is thought to be mediated by a novel adaptor protein CBL (Casitas B-lineage lymphoma) (Wang et al., 2004).

1.4.2.5 Flk-1 signalling during development and in developmental models

The importance of Y1173 (which has been demonstrated to bind PLC- γ , Shb and PI3K in KDR) was demonstrated in a knock-in mouse in which tyrosine 1173 (Y1175 KDR) in the cytoplasmic tail of Flk-1 was substituted for a phenylalanine residue. The mutation resulted in early embryonic lethality at E8.5-9.5 resembling the Flk-1 knockout mouse suggesting that Y1173 is key in transducing Flk-1 signalling at this time (Sakurai et al., 2005). Shb itself may also play an important role in development. An ES cell line expressing an SH2 mutant of Shb displayed a greatly decreased ability for blood vessel formation in an embryoid body model suggesting it plays a crucial role, at least *in vitro*, in the differentiation of vascular structures (Rolny et al., 2005).

Little investigation of signalling downstream of Flk-1 during development has been performed. 30 minute VEGF stimulations of embryoid body-derived cells expressing Flk-1 demonstrated activation of the MAPK pathway, but not the PI3K pathway (Park et al., 2004). However, both pathways are known to be activated transiently by VEGF in adult endothelial cells and the VEGF-induced increase in phosphorylation of down-stream mediators in both pathways is often decreased or abolished by the 30 minute time point making it difficult to draw definitive conclusions from this study (Bernatchez et al., 2001; Dayanir et al., 2001; Gerber et al., 1998; Takahashi and Shibuya, 1997). In zebrafish, inhibition of Flk-1 and Flt-1 with small molecule inhibitor 676475, greatly reduced ERK phosphorylation analysed using immunoblotting or whole-mount embryo immunostaining, suggesting a role for VEGF signalling in the activation of the MAPK pathway (Hong et al., 2006).

1.5 Aims

There has been much research into KDR (human Flk-1) signalling in adults as KDR mediates VEGF-induced angiogenesis and is therefore important in a number of pathological conditions including cancer. The Flk-1 knockout in mice is embryonic lethal by E9.5 due to a lack of formation of vasculature or blood (Shalaby et al., 1995; Shalaby et al., 1997) demonstrating that Flk-1 signalling also plays a key role in embryonic development, specifically development of the vasculature and blood. However, despite extensive knowledge of adult KDR signalling little is known about the signalling pathways activated by Flk-1, a receptor tyrosine kinase, during development, and the importance of these individual signalling pathways in haemopoietic and endothelial differentiation. The overall aim of this project was therefore to study the Flk-1 generated signals involved in ES cell differentiation to haemopoietic, endothelial and cardiomyocyte cell lineages. Specific objectives of the project included:

- Elucidation of the intracellular signals activated by VEGF, in murine ES cells, during differentiation. The signalling pathways of particular interest were those activated by VEGF in adult endothelial cells which include the MAPK pathway, the PI3K pathway and other signalling and adaptor molecules which associate with Flk-1 (Dayanir et al., 2001; Doanes et al., 1999; Dougher and Terman, 1999; Gille et al., 2001; Glikli et al., 2001; Guo et al., 1995; Kroll and Waltenberger, 1997; Meyer et al., 2003; Takahashi et al., 2001; Wu et al., 2000a).
- To use Flk-1 null ES cells and specific inhibitors to investigate the importance of various Flk-1-mediated signals in haemopoietic, endothelial and cardiac specification.
- To generate an ES cell line upon the background of the Flk-1 null ES cell line (obtained from Professor William Stanford, University of Toronto) which conditionally express Flk-1. The aim was to use this cell line to investigate the temporal importance of Flk-1 in haemopoietic, endothelial and cardiac differentiation.

Chapter 2

Materials & methods

2.1 Cell lines and tissue culture

2.1.1 Cell lines

2.1.1.1 E14tg2A murine embryonic stem cell line

The E14tg2A murine embryonic stem cell line (clone R63) was a kind gift of Dr. Owen Witte, UCLA, California (Era and Witte, 2000). The E14tg2A cell line is stably transfected with a tetracycline-regulated transactivator construct pCAG20-1.

2.1.1.2 R1 embryonic stem cell line

The R1 murine embryonic stem cell line was a kind gift of Professor William Stanford, University of Toronto, Ontario (Nagy et al., 1993)

2.1.1.3 $Flk-1^{-/-}$ embryonic stem cell line

The $Flk-1^{-/-}$ murine embryonic stem cell line was a kind gift of Professor William Stanford, University of Toronto, Ontario. $Flk-1^{-/-}$ ES cells were originally derived from $Flk-1^{+/-}$ cells which were produced from the R1 parentals by gene-targeting of the *lacZ* gene into the first exon of *Flk-1* placing it under the transcriptional control of *Flk-1* regulatory elements (Shalaby et al., 1995). Selection in high concentrations of G418 allowed generation of the homozygous knockout (Shalaby et al., 1997).

2.1.1.4 D4T

D4T is an embryonic stem cell derived endothelial cell line, a kind gift of Dr. Georges Lacaud, Paterson Institute, University of Manchester, UK.

2.1.1.5 Human Umbilical Vascular Endothelial Cells (HUVECs)

HUVECs were isolated by Dr. James Hewison, with appropriate ethical and parental consent, from human umbilical veins of new born babies (Jaffe et al., 1973).

2.1.1.6 OP9

OP9 is a murine macrophage colony-stimulating factor-deficient bone marrow-derived stromal cell line which is commonly used to support haemopoietic differentiation (Nakano, 1995).

2.1.1.7 BaF/3

An immortalised murine bone marrow derived pro-B cell line dependent on interleukin-3 (IL-3) for proliferation (Palacios and Steinmetz, 1985).

2.1.2 Tissue culture

All cells were maintained at 37°C in a humidified incubator with 5% (v/v) CO₂. Cell counts were performed using a haemocytometer.

2.1.2.1 Embryonic stem cell culture

Embryonic stem cell lines (R1, E14tg2A and Flk-1^{-/-}) were routinely cultured on 92 x 17mm NUNC dishes coated with 0.1% (w/v) porcine gelatine in knockout Dulbecco's Modified Eagle Medium (DMEM) supplemented with 15%(v/v) knockout serum replacement, 2mM Glutamine, 50µM 2-mercaptoethanol, 1% non-essential amino acids and 1000U/ml Leukaemia inhibitory factor (LIF), or 4ul/ml recombinant LIF conditioned media (batch tested in house in self-renewal and stimulation experiments) from the HEK293LIFV5 cell line (Park et al., 2003) generated by stable expression of a V5 epitope-tagged LIF gene in HEK293 cells (a kind gift of Dr. Konstantinos Anastassiadis, University of Technology, Dresden). 0.1% (v/v) Hyclone serum (Perbio, Hyclone, UK) was used to supplement media of R1 and Flk-1^{-/-} ES cells. The cultures were passaged by application of ~0.5ml Trypsin/Ethylenediaminetetraaceticacid (EDTA) for 5 minutes at 37°C and mechanical agitation to dissociate the cells. Cell passage was performed every two to three days to keep cell density sub-confluent (< 1.5x10⁶ cells per dish) thus preventing differentiation. Cultures were passaged for no longer than 3 weeks (Paling et al., 2004).

2.1.2.2 D4T

D4T cells were routinely cultured on T75 tissue culture flasks coated with 0.1% (w/v) porcine gelatine. They were maintained in Iscoves' Modified Eagle Medium (IMDM) supplemented with 10% ES cell differentiation tested Foetal Calf Serum (FCS), 50ng/ml endothelial cell growth supplement (ECGS) and 150 μ M monothioglycerol (MTG). Cells were grown to confluence and passaged 1 in 3 by application of trypsin/EDTA and mechanical agitation.

2.1.2.3 Human Umbilical Vein Endothelial Cells

Human umbilical vein endothelial cells (HUVECs) were isolated by Dr. James Hewison from human umbilical veins of newborn babies by digestion with collagenase at 1mg/ml in Hanks Balanced Salt Solution (HBSS) for 45 minutes and cultured in endothelial cell growth medium for up to 4 passages .

2.1.2.4 OP9 cells

OP9 cells were cultured on NUNC tissue culture dishes in α -Modified Eagle Medium (α -MEM) supplemented with 20% (v/v) FCS, 2mM glutamine and 50 μ M 2-mercaptoethanol. Cells were grown to confluence and passaged 1 in 4 by application of trypsin/EDTA and mechanical agitation.

2.1.2.5 BaF/3 cells

BaF/3 cells were cultured in non-tissue-culture treated plastic in RPMI 1640 medium supplemented with 10% (v/v) FCS, 20 μ M 2-mercaptoethanol, 100U penicillin/streptomycin, 2mM glutamine and 5-10% JWW3/WEH1 3B conditioned media cells as a source of IL-3. Cells were regularly passaged by serial dilution

2.1.3 Freezing/thawing cells

For storage, cells were gently resuspended in cold freezing media as follows;

Basic freezing media

Glasgow modified eagle medium (GMEM) supplemented with 2mM glutamine, 50µM 2-mercaptoethanol, 1% (v/v) non-essential amino acids, 1mM sodium pyruvate.

ES cells: 1×10^6 cells/ml in basic freezing media supplemented with 10% (v/v) dimethylsulphoxide (DMSO) and 10% ES screened foetal bovine serum (FBS)

D4T cells: One confluent T75 flask into 3ml basic freezing media supplemented with 40% FCS and 10% DMSO

OP-9 cells: 1×10^6 /ml in basic freezing media supplemented with 40% FCS and 10% DMSO.

BaF/3 cells: $\geq 2 \times 10^6$ /ml in 90% FCS and 10% DMSO

The cells were then aliquoted into 1ml nunc cryovials and placed at -80°C overnight before being transferred for long term storage in liquid nitrogen. For recovery, cells were thawed rapidly at 37°C, gently resuspended in 10ml of appropriate growth media and pelleted at 1000r.p.m. (~140g) for 5 minutes in a Jouan CR412 centrifuge before resuspension in complete media.

2.1.4 Screening cells for mycoplasma

Mycoplasma are a relatively common bacterial contaminant of cultured cell lines. The bacteria have no cell wall and are therefore resistant to most antibiotics. They are difficult to visualise under the microscope due to their small size but their presence is deleterious and can skew experimental results (Macpherson, 1966). To detect any potential mycoplasma contamination cells were screened for mycoplasma using the Stratagene Mycosensor PCR assay kit. Briefly cell culture supernatant was

boiled with Stratagene resin to remove cell culture components such as metabolites and FCS. This boiled supernatant was then used as the PCR template in a PCR reaction (2.4.3.4) designed to amplify a 315bp band using a mycoplasma primer mix. Positive and negative controls and an internal control template were also included. The resultant products were run on a 2% agarose gel (2.4.2.3) to check for mycoplasma contamination. No mycoplasma contamination was found in any of the cell lines used in this study.

Table 2-1 Tissue culture consumables

Product	Supplier	Cat. number
Growth medium		
α -Modified Eagle Medium (α -MEM)	Invitrogen, Paisley, UK.	22571-020
Endothelial cell growth medium	Promocell, Heidelberg, Germany.	C-22010
Glasgow's Modified Eagle Medium (GMEM)	Invitrogen	21710-025
Iscove's Modified Eagle Medium (IMDM)	Invitrogen	21980-032
knockout Dulbecco's Modified Eagle Medium (DMEM)	Invitrogen,	10829-018
RPMI 1640	Invitrogen	21875-059
Medium supplements		
endothelial cell growth supplement (ECGS)	R&D systems, Abingdon, UK	231-BC-025
ESGRO/LIF	Chemicon, Hampshire, UK.	ESG1106
Fetal Calf Serum – BaF/3	Sigma, Dorset, UK.	
Fetal Calf Serum – D4T and OP-9	Invitrogen	16000-044 lot#1168507
200mM L-Glutamine	Invitrogen	25030-024
Hyclone Fetal Calf serum - ES cells	Perbio, Hyclone, UK.	SH30070.03E
Knockout serum replacement	Invitrogen	10828-028
2-mercaptoethanol (2-ME)	Bio-Rad, Hemel Hempstead Hertfordshire	161-0710
Monothioglycerol (MTG)	Sigma	M6145
100X Non-essential amino acids (NEAA)	Invitrogen	11140-050
Penicillin/streptomycin	Invitrogen	15140-122
Porcine gelatin	Sigma	G1890
Other reagents		
Collagenase type II	Sigma	C-6885
DMSO	Sigma	D2650
10 X Hanks Balanced Salt Solution (HBSS)	Invitrogen	14060-040
Mycosensor PCR kit	Stratagene, Amsterdam, The Netherlands.	302109

Phosphate Buffered Saline (PBS)	Invitrogen	14200-067
Trypsin/ Ethylenediaminetetraaceticacid (EDTA)	Invitrogen	25200-072
Tissue culture plastic ware		
15ml centrifuge tubes	Greiner Bio-one (GBO), Gloucestershire, UK.	188271
50ml centrifuge tubes	GBO	227261
NUNC cryovials	Fisher Scientific, Leicestershire, UK.	CRY-960-070B
NUNC tissue culture dish 60 x 15mm	Fisher	TKT-110-010S
NUNC tissue culture dish 92 x 17mm	Fisher	TKT-110-070A
NUNC T25 tissue culture flask	Fisher	TKT-130-050P
NUNC T75 tissue culture flasks	Fisher	TKT-130-130R
3ml Pasteur Pipettes	GBO	612398
Petri dishes 60 x 15mm	GBO	628160
Petri dishes 100 x 20mm	GBO	664102
10ml Pipettes, Sterile, Single Wrapped	GBO	607180
25ml Pipettes, Sterile, Single Wrapped	GBO	760180
150mm unplugged glass pipette	Fisher	FB50251

2.2 ES differentiation protocols

The development of embryoid bodies (three dimensional aggregates of embryonic stem cells) mimics early embryogenesis and can be used to dissect the formation of particular cell lineages (see section 1.2.2 for additional detail). The main uses of embryoid bodies in this study are illustrated in Figure 2-1 below.

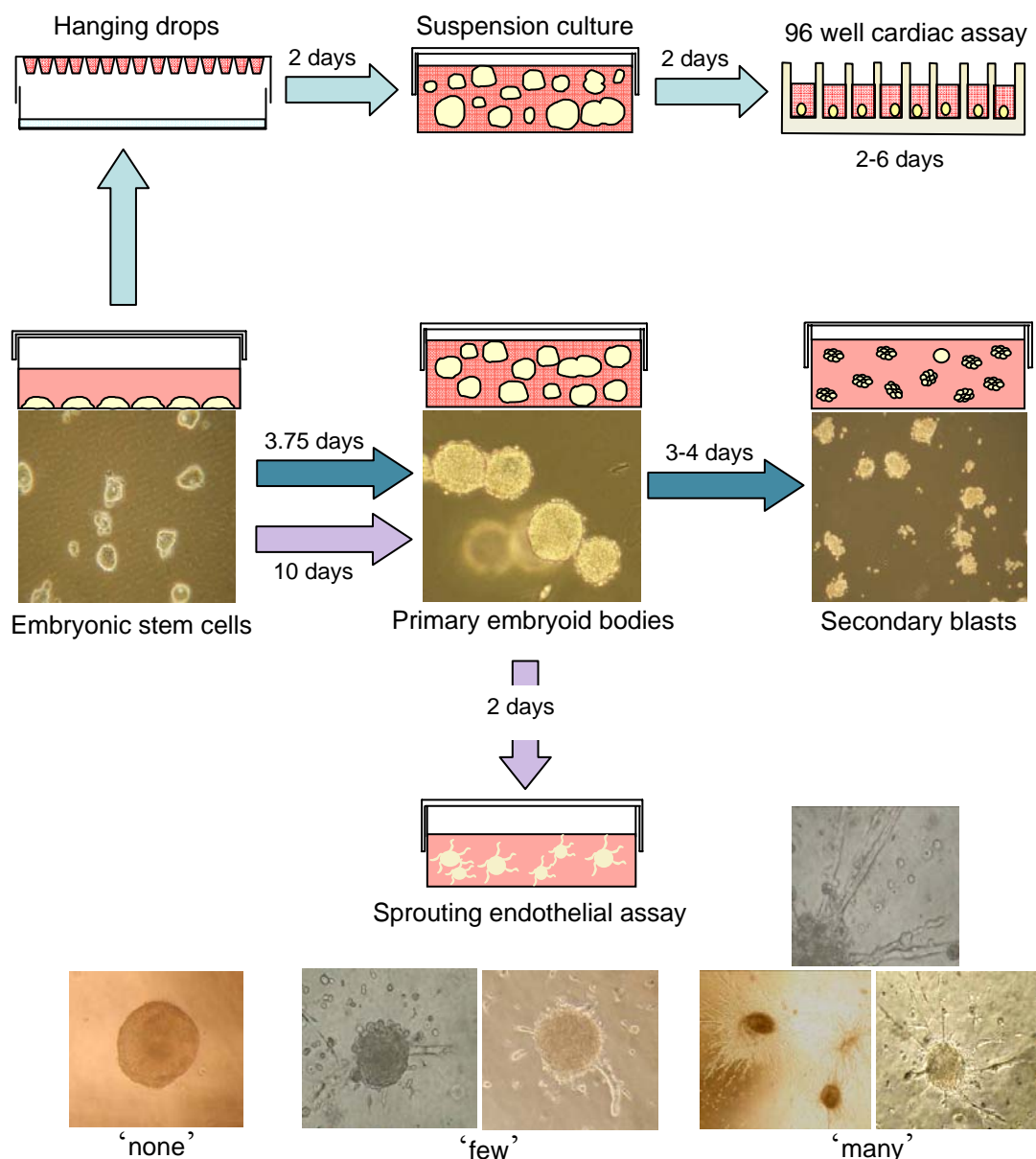


Figure 2-1 Illustration of some of the models of development used during the study

2.2.1 Primary embryoid body culture

The primary embryoid body formation protocol was adapted from Kennedy *et al.*, 1997 and Keller *et al.*, 1993. The cytokines added have been shown to promote differentiation towards the mesodermal lineage.

For formation of embryoid bodies ES cells were resuspended at 1×10^4 /ml in IMDM and either 40% ES-Cult or 50% of the in house equivalent (2% methylcellulose plus 1.2% sodium bicarbonate in IMDM). This was supplemented with 15% FCS screened for its ability to support ES cell differentiation to form secondary blast colonies (ES FCS), 200µg/ml *holotransferrin* (Kennedy *et al.*, 1997), 450µM MTG, 50µg/ml L-ascorbic acid (Kennedy *et al.*, 1997; Robertson *et al.*, 2000), 10µg/ml recombinant human insulin (Johansson and Wiles, 1995; Keller *et al.*, 1993), 10ng/ml basic fibroblast growth factor (bFGF) (Faloon *et al.*, 2000) and 2ng/ml recombinant human Activin A (Faloon *et al.*, 2000; Johansson and Wiles, 1995). The culture mixture was vortexed and aliquotted into 30mm petri dishes, two of which were incubated with one 30mm dish of water in a 100mm petri dish at 37°C in a humidified atmosphere of 5% (v/v) CO₂.

2.2.2 Secondary blast formation

Secondary blast formation is an expansion and further differentiation of the Blast Colony Forming Cell (BL-CFC) the *in vitro* equivalent of the haemangioblast, and as such can be used as a measure of its formation as well as a tool to further study mesodermal differentiation. A cell density of at least 5×10^4 /ml is important for efficient blast formation though the presence of D4T conditioned media (Kennedy *et al.*, 1997) does alleviate this to some extent. The stage at which embryoid bodies are dissociated and replated into the secondary blast assay is also important and differs between ES cells lines. It is important to dissociate primary embryoid bodies for replating of their constituent cells into the secondary blast assay during the narrow temporal window of BL-CFC formation which is marked by upregulation of Flk-1 (Fehling *et al.*, 2003; Huber *et al.*, 2004). In E14tg2A cells this occurs between days 3.5 and 4.

To expand mesodermal cells from the first round of embryoid body formation, day 3.75 primary embryoid bodies were washed off the culture dishes with Phosphate buffered saline (PBS) then washed a further two times in 50ml of PBS at 1500r.p.m. (~350g) for 5 minutes to remove remaining methylcellulose. Embryoid bodies were then dissociated in 2ml of Trypsin/EDTA for 3 minutes at 37°C and the Trypsin/EDTA was neutralised with 2ml ES FCS. The cells were washed in PBS (5 minutes at ~140g) before being re-suspended at 5×10^4 /ml in IMDM with 40% ES-Cult, 10% ES FCS, 25% D4T conditioned media, 450µM MTG, 5ng/ml recombinant murine vascular endothelial growth factor (mVEGF) (Kennedy et al., 1997), 200µg/ml *holotransferrin*, 25µg/ml L-ascorbic acid, and 5ng/ml recombinant murine interleukin-6 (IL-6) (Mikkola et al., 2003). The culture mixture was vortexed and aliquoted as before into 30mm petri dishes, two of which were incubated with one 30mm dish of water in a 100mm petri dish at 37°C in a humidified atmosphere of 5% (v/v) CO₂.

2.2.3 Sprouting endothelial assay

The sprouting endothelial assay was performed using Stem Cell Technologies ES-Cult Endothelial Collagen and Medium protocol. Firstly, ES cells were differentiated into embryoid bodies by seeding at 1.34×10^3 /ml in IMDM supplemented with 40% ES-Cult, 15% ES FCS, 10µg/ml rh-insulin, 450µM MTG, 50ng/ml mVEGF, 100ng/ml bFGF, 10ng/ml IL-6 and 2U/ml recombinant human erythropoietin (EPO). The mixture was aliquotted into 30mm bacterial Petri dishes in 1.5ml aliquots using a 2ml syringe and 18G blunt needle. Two Petri dishes were incubated with one 30mm Petri dish of water in a 100mm bacterial Petri dish at 37°C in a humidified atmosphere of 5% (v/v) CO₂. After 10-11 days the embryoid bodies were counted and gently washed off the dishes with IMDM. They were washed twice in IMDM (1000 r.p.m for 5 minutes) and resuspended at 2500EBs/ml in IMDM. 0.1ml of EBs was then added to 1.7ml of ES-Cult endothelial basal medium and 0.3ml of IMDM supplemented with 50ng/ml mVEGF, 100ng/ml bFGF, 10ng/ml IL-6 and 2U/ml EPO and gently swirled to mix. 1.2ml of collagen solution was added and pipetted to mix before 1.5ml was aliquotted into each of two 30mm bacterial Petri dishes using a p1000 Gilson pipette. As before two 30mm culture dishes were placed with one

30mm dish of water in a 100mm bacterial petri dish and incubated at 37°C in a humidified atmosphere of 5% (v/v) CO₂.

2.2.4 Haemopoietic plaque assay

The haemopoietic plaque assay was adapted from Hidaka et al., 1999; Nakano et al., 1994; Stanford et al., 1998 . A 6 well dish of semi-confluent OP-9 cells was seeded with 1×10^4 undifferentiated ES cells per well in OP-9 medium (α -MEM supplemented with 20% (v/v) FCS, 2mM glutamine and 50 μ M 2-mercaptoethanol) and cultured at 37°C in a humidified atmosphere of 5% (v/v) CO₂ for 5 days to induce formation of mesodermal plaques. After 5 days trypsin/EDTA was added to dissociate the plaques (and OP9s). Following neutralisation of the trypsin/EDTA with ES FCS the cells were resuspended in OP9 medium and centrifuged for 5 minutes at ~140g. The washed cells were plated onto 10cm NUNC dishes (3 x 10cm dishes/6well plate) and incubated for 30 minutes at 37°C to allow adhesion of the OP-9s. Non-adherent cells were counted and replated at 1.2×10^5 /well onto a fresh 6 well dish of confluent OP9s for a further 7 days to induce differentiation of haemopoietic plaques.

2.2.5 Cardiomyocyte assay

The cardiomyocyte assay was adapted from (Sachinidis et al., 2003a) and a method provided by the FUNGENES EU consortium. ES cells were resuspended at 2.5×10^4 /ml in IMDM supplemented with 20% ES FCS and 450 μ M MTG. 20 μ l drops of the cell suspension were pipetted onto the lids of 100mm bacterial petri dishes which were then inverted and replaced on the petri dishes over 7ml of PBS. The dishes were incubated in a humidified incubator with 5% CO₂ at 37°C for two days. After two days the drops were aspirated off the lids of the petri dishes using a p1000 pipette and re-plated in 10ml of fresh media in a 100mm bacterial Petri dish. Following a further two day incubation period, individual embryoid bodies were placed in fresh media into wells of a 96 well plate which were coated in 0.2% (w/v) gelatine. Individual wells were scored for the appearance of beating areas of cells indicative of cardiomyocyte formation daily from 2 to 6 days later. Media was changed as necessary.

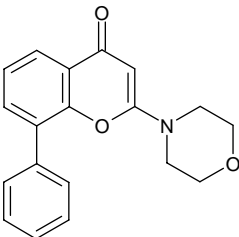
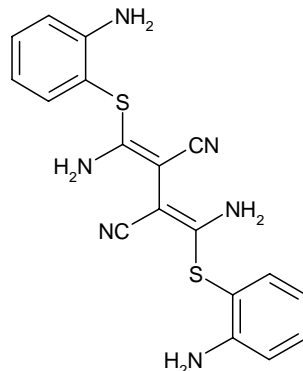
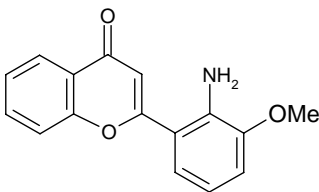
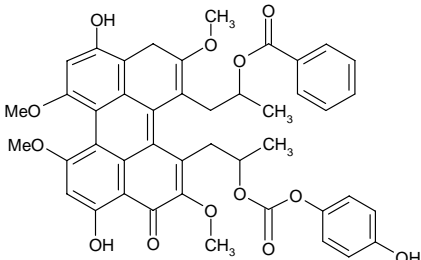
Table 2-2 Differentiation protocol consumables

Product	Supplier	Cat. number
Growth media		
α -MEM	Invitrogen	22571-020
ES Cult endothelial collagen and medium	Stemcell Technologies Inc, Canada.	05810
ES-Cult M3120 base methylcellulose	Stemcell Technologies	03120
1X IMDM	Invitrogen	21980-032
IMDM (powder)	Invitrogen	42200-014
Methylcellulose	Sigma	M0512
Medium supplement		
rhActivin A	R & D systems	338-AC-005
L-ascorbic acid	Sigma	A4544
bFGF	Peptrotech, London, UK.	100-18B
rhEPO	R & D systems	287-TC-500
FCS	Invitrogen	16000-044 (lot #1168507)
200mM L-Glutamine	Invitrogen	25030-024
rmIL-6	Peptrotech	216-16B
rhInsulin	Sigma	19278
2-mercaptoethanol	Bio-Rad	161-0710
MTG	Sigma	M6145
Sodium Bicarbonate	Invitrogen	25080-060
<i>holo</i> -transferrin	Sigma	T-0665
mVEGF	Peptrotech	450-32B
Other reagents		
Trypsin/EDTA	Invitrogen	25200-072
PBS	Invitrogen	14200-067
Tissue culture plastic ware		
18G blunt needle	Fisher	SZR-175-587L
15ml centrifuge tubes	GBO	188271
50ml centrifuge tubes	GBO	227261
NUNC 6 well tissue culture dish	Fisher	TKT-190-110E
NUNC tissue culture dish 92 x 17mm	Fisher	TKT-110-070A
3ml Pasteur Pipettes	GBO	612398
Petri dishes 30 x 10mm	Fisher	PDS-140-170S
Petri dishes 60 x 15mm	GBO	628160
Petri dishes 100 x 20mm	GBO	664102
Petri dishes (non tissue culture)	Fisher	PDS-140-050F
10ml Pipettes, Sterile, Single Wrapped	GBO	607180
25ml Pipettes, Sterile, Single Wrapped	GBO	760180
2ml syringe	Central Stores	
150mm unplugged glass pipette	Fisher	FB50251

2.3 Inhibitors used in the study

Details of inhibitors used to study signalling pathways activated by Flk-1 and their functional importance in *in vitro* differentiation protocols are given in table 2-3 below.

Table 2-3 Inhibitors used in study

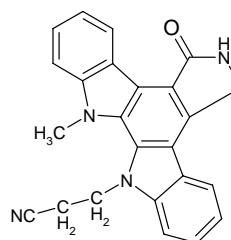
Inhibitor name and selected reference	Target of inhibitor	Inhibitor structure	Calbiochem catalogue number
LY294002 2-(4-morpholinyl)-8-phenyl-4H-1benzopyran-4-one	PI3K		440202
(Vlahos et al., 1994) U0126 1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene	MEK1/2		662005
(Favata et al., 1998) PD98059 2'Amino-3'-methoxyflavone	MEK1		513000
(Alessi et al., 1995) Calphostin UCN-1028c (Kobayashi et al., 1989)	PKC		208725

Gö6976

12-(2-Cyanoethyl)-
6,7,12,13-tetrahydro-13-
methyl-5-oxo-5H-
indolo(2,3-a)pyrrolo(3,4-
c)-carbazole

PKC α ,
 β_1, β_2 , and
PKD

36525



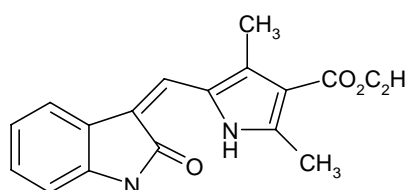
(Martiny-Baron et al.,
1993)

VEGFR2 kinase
inhibitor-1

Flk-1

676480

(z)-3-[(2,4-Dimethyl-
3(ethoxycarbonyl)pyrrol-
5yl)methylidenyl]indolin-
2-one

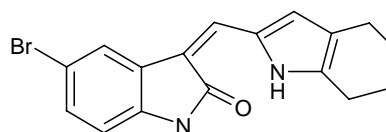


(Sun et al., 1998)
VEGFR2 kinase
inhibitor-2

Flk-1

676485

z-5-Bromo-
3(4,5,6,7-tetrahydro-1H-
indol-2-ylmethylene)-1,2-
dihydroindol-2-one



(Sun et al., 2000)

2.4 Biochemical and functional techniques

2.4.1 Cell stimulation and preparation of cell extracts

Dissociated embryoid bodies were stimulated with VEGF to allow investigation of Flk-1 (VEGF receptor 2) coupled signalling pathways using immunoblotting.

Embryoid bodies were dissociated (see section 2.2.2), washed three times in PBS (5 minutes at 1000r.p.m.) and re-suspended at $1-2 \times 10^6$ /ml in Glasgow modified eagle media (GMEM/BHK-21) supplemented with 2mM Glutamine, 50 μ M 2-mercaptoethanol, 1% (v/v) non-essential amino acids and 20mM HEPES. The cells

were serum starved for 3 hours at 37°C then stimulated with 20ng/ml recombinant murine vascular endothelial growth factor (VEGF) (Peprotech) for appropriate time course at 37°C. Following stimulation cells were pelleted at full speed (~20800 x g) in an eppendorf 5417R refrigerated microcentrifuge. The supernatant was aspirated and the pellet lysed in ~30-50µl per 10⁶ cells of cold solubilisation buffer (50mM Tris-HCl pH7.5, 150mM Sodium Chloride, 1% (v/v) Nonidet P40, 10% (v/v) glycerol, 5mM EDTA, 1mM Sodium Vanadate, 1mM Sodium Molybdate, 10mM Sodium Fluoride, 40µg/ml Phenylmethylsulphonylfluoride (PMSF), 0.7µg/ml Pepstatin A, 10µg/ml Aprotinin, 10µg/ml Leupeptin, 10µg/ml soyabean trypsin inhibitor. The lysates were spun down for 2 minutes at full speed to pellet nuclear debris and the supernatants were stored at -20°C. Where the effect of a small molecule inhibitor was under investigation this was added at the required concentration 30 minutes prior to stimulation.

2.4.2 Bradford assay

Protein concentrations in whole cell lysates were determined using the Bradford assay (Bradford, 1976) which utilises an absorption shift in Coomassie brilliant blue dye at 595nm when bound to arginine and hydrophobic residues in a protein. The relationship between this absorption shift and the amount of protein present is linear in the range 2-120µg/ml. Generation of a standard curve using between 2-12µg of BSA mixed well in 1ml of 1 in 5 Bradford Reagent was used to extrapolate protein concentrations in diluted whole cell lysates. 100µl of each sample was placed in duplicate wells of a clear, flat bottomed 96 well plate and read at 595nm using a Versamax microplate reader (Molecular Devices). Lysates were diluted as required to equalise sample concentrations determined by Bradford assay and boiled in SDS-sample buffer (5X sample buffer; 10% SDS (w/v), 50% glycerol (v/v) 200mM Tris-HCl pH 6.8, 5% 2-mercaptoethanol and ~ 2% bromophenol blue in milliQ water).

2.4.3 Protein resolution and immunoblotting

2.4.3.1 *Sodium-dodecyl-sulphate Polyacrylamide gel electrophoresis (SDS-PAGE)*

SDS-PAGE is a technique by which proteins are separated according to their mass and charge by application of a current. The protein sample or cell extract is boiled in a buffer containing 2-mercaptoethanol which denatures quaternary structures and disulphide linkages and SDS, an ionic detergent which denatures the secondary and non-disulphide structures of the protein and coats the linearised protein. SDS binds at a rate of 1.4g SDS per 1g Protein which gives all SDS-bound proteins roughly the same charge to mass ratio, and hence allows separation of proteins by polypeptide size alone. The samples are loaded into a gel matrix containing a lower resolving gel and upper stacking gel in which the proteins are focussed into a single sharp band. The stacking gel is a non-restrictive large pore matrix with a pH of 6.8, that of the Tris-glycine reservoir buffer. At pH 6.8 glycine is weakly ionised and has low mobility but NaCl also present in the buffer is fully ionised and has greater mobility upon application of a current. The protein sample is of intermediate mobility. These differences in electrophoretic mobility allow formation of a NaCl/glycine boundary which migrates through the stacking gel concentrating the proteins into a focussed band. The small pore resolving gel has a pH of 8.8 at which glycine is strongly ionised and has greater effective mobility migrating through the proteins and allowing their separation (Laemmli, 1970).

The gels were prepared using Bio-Rad's mini Protean II and III gel electrophoresis equipment assembled according to the manufacturer's guidelines. The percentage of acrylamide used in the resolving gel was dependent upon the size of the target protein/s (see Table 2-4 below):

Table 2-4 Resolving power of differing acrylamide percentages in PAGE

% acrylamide	Size of target protein (kDa)
6	60-250
7.5	40-200
10	25-200
12	15-100

The resolving gel was prepared at the required percentage of acrylamide and ~ 4.5 ml was poured into the gel casting apparatus and carefully overlaid with ddH₂O (for details of gel composition see Table 2-5).

Table 2-5 Acrylamide resolving gel compositions

% acrylamide	Bis/Acrl (ml)	Milli-Q H ₂ O (ml)	1M TRIS- HCL pH 6.8 (ml)	10% (w/v) SDS (ml)
6.0	3.0	6.35	5.6	0.25
6.5	3.25	6.1	5.6	0.25
7.0	3.5	5.85	5.6	0.25
7.5	3.75	5.6	5.6	0.25
8.0	4.0	5.35	5.6	0.25
10	5.0	4.35	5.6	0.25
12	6.0	3.35	5.6	0.25
15	7.5	1.85	5.6	0.25

plus 50µl 10% (w/v) Ammonium persulphate (APS) and 20µl Tetramethylethylenediamine (TEMED) for acrylamide cross-linking reaction

Following aspiration of the ddH₂O from above the set resolving the stacking gel (1.67ml Acrylamide/Bisacrylamide solution, 6.0ml milliQ water, 1.25ml 1M Tris-HCl pH 6.8, 0.15ml 10% (w/v) SDS plus 50µl 10% (w/v) APS and 20µl TEMED) was layered on top and a well forming comb inserted. Between 5 and 15µl of sample was loaded into each lane. 10µl of 1% (v/v) SDS-PAGE broad range molecular weight marker (biorad) made up in 1x SDS sample buffer and boiled was also loaded into one lane on each gel. The gels were run at 80mV to stack and 160mV to resolve in 1x running buffer (10x running buffer; 3%(w/v) Tris, 1%(w/v) SDS, 14.4% (w/v) glycine in milliQ water).

2.4.3.2 Immunoblotting

Immunoblotting or Western Blotting transfers protein from SDS-PAGE gels onto nitrocellulose paper to allow detection of proteins using dyes or antibody based detection systems (Burnette, 1981).

Following gel electrophoresis, the separated proteins were transferred onto nitrocellulose membranes by semi-dry transfer using the Pharmacia LKB Novoblot electroblotting equipment. The graphite electrodes of the semi-dry transfer apparatus were dampened with semi-dry transfer buffer (39mM glycine 48mM Tris base, 1.3mM SDS, 20% (v/v) methanol in milliQ water) and the transfer sandwich was assembled on the lower electrode as follows;. four sheets of 8.5x5.2cm 3MM Whatman chromatography paper, nitrocellulose blotting membrane of the same size, the SDS-PAGE gel and finally four more pieces of Whatman paper all soaked in semi-dry transfer buffer. All air bubbles were expelled and transfer was performed at 40mA/gel for 1 hour. Following transfer, the nitrocellulose was stained with Ponceau S solution to allow visualisation and marking of the SDS-PAGE broad range molecular markers on the blot. The Ponceau was rinsed off with Tris-buffered saline (TBS) (10x TBS; 1.5M NaCl, 0.2M Tris in 2l, pH7.5 with HCl) and the sites on the membrane which transferred proteins had not bound were ‘blocked’ with one of the following protein blocks for 1 hour on a rocker at room temperature; 5% block (5% (w/v) Bovine serum albumen (BSA), 1% (w/v) ovalbumen, 0.05%(w/v) sodium azide in TBS); 5% MARVEL powdered milk in TBS or 2% ECL advance blocking solution depending upon the antibody used on a rocker at room temperature. Excess block was washed off and blots were incubated with primary antibody at 4°C for 3 – 16 hours (see Table 2-6).

Table 2-6 Antibody list

Antibody	Source	Blocking conditions	Primary antibody		
			Diluent	Dilution	Incubation period (hours)
Primary antibodies					
4G10	mouse	5% block	1% block	0.1µg/ml	3
α-phospho-ERK1/2	rabbit	5% block	1% block	1:1000	16
α- ERK1/2	Rabbit	5% block	1% block	1:1000	16
α-phospho-Flk-1*	Rabbit	ECL advance block	1% block	1:500	16
α- Flk-1*	Rabbit	ECL advance block	1% block	1:500	16
α-phospho-	Rabbit	5% block	1% block	1:1000	16

GSK-3 α/β					
α - GSK-3 β	Rabbit	5% block	1% block	1:1000	16
α -p85 (PI3K)	rabbit	5% block	1% block	1:1000	3
α -phospho- PKB/Akt	Rabbit	5% block	0.3% block	1:1000	16
α - PKB/Akt	Rabbit	5% block	1% block	1:1000	16
α -phospho- PKB/Akt substrate	Rabbit	5% block	0.3% block	1:1000	16
α -phospho-PKC (pan)	Rabbit	5% milk 0.1% Tween	3% block	1:1000	16
α -SHP-2	Rabbit	5% block	1% block	1:2000	16
α -phospho-S6	Rabbit	5% block	1% block	1:1000	3
Secondary antibodies					
Mouse-HRP	Goat	-	TBS, 0.1% Tween	1:10000	1-2
Rabbit-HRP	Goat	-	TBS, 0.1% Tween (*1:5000)	1:20000	1-2

The blots were then washed with one TBS wash followed by three TBST (TBS with 0.05%v/v Tween) washes and a final TBS wash and incubated with an appropriately diluted horse radish peroxidase (HRP)-conjugated secondary antibody made up in TBSN (TBS with 0.05% (v/v) NP40) for 1-2 hours. The blots were washed as before with an additional final TBS wash to remove remaining detergent then developed in ECL or ECL advanced chemiluminescent solutions according to the manufacturers' protocol. The resultant ECL signal was visualised by exposure of the membrane to Kodak X-AR5 film and developed in a dark room using a RGII Fuji X-Ray film developer.

2.4.3.3 Stripping and re-probing immunoblots

Blots to be re-probed with a different antibody were rinsed in TBS then stripped for 45 minutes at 55°C in stripping buffer (6.25% (v/v) 1M Tris-HCl pH 7.5, 2%(w/v) SDS, 0.77% (v/v) 2-mercaptoethanol). Following stripping blots were rinsed thoroughly in TBSN (2-mercaptoethanol waste was disposed of in the appropriate waste bottle) and blocked for 1 hour in used 5% block. The block was rinsed off and the blots were re-probed as before.

2.4.4 Fluorescence activated cell sorting (FACS) (flow cytometry)

2.4.4.1 Overview of flow cytometry

During this study flow cytometry was used for molecular analysis of cell surface expression of Flk-1. Flow cytometry utilises a focussed beam of laser light projected through a liquid stream of single cells or other particles. Photomultiplier Tubes detect fluorescence emission and scattering of the light by the cells and extrapolate their relative size, granularity and fluorescence. A standard flow cytometer is illustrated in Figure 2-2.

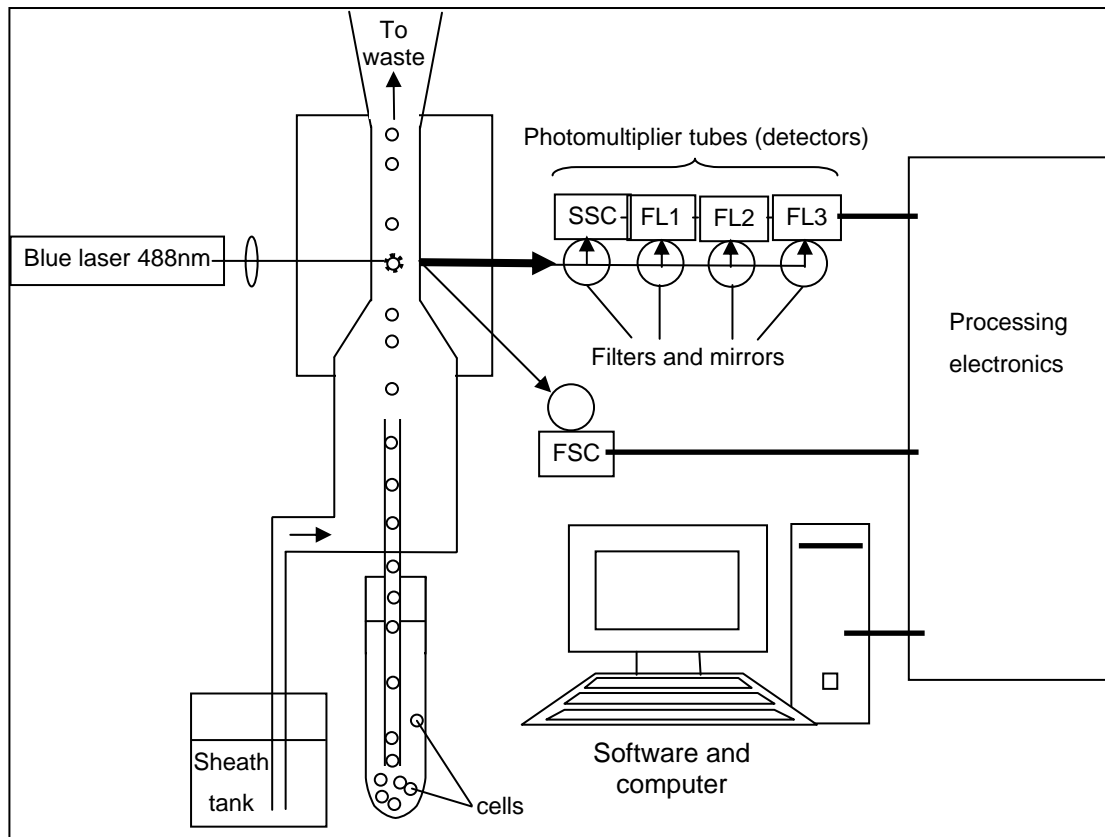


Figure 2-2 Diagram of a simple flow cytometer. A stream of individual cells flows through a laser beam at up to 1000 cells (events) per second. Diffracted, refracted, reflected and emitted light is detected in photomultiplier tubes and the light signals are converted into electronic signals for analysis and interpretation. Adapted from ‘introduction to flow cytometry’ from Becton Dickinson online accessed 21st March 2007. http://www.bdbiosciences.com/immunocytometry_systems/support/training/online/ITF/start.html.

The basic physical properties of cell size and cell granularity or complexity are measured using the forward scatter (defracted light) and side scatter (refracted and reflected light) as illustrated in Figure 2-3. These properties can be used to separate different populations of cells in a mixed cell population (e.g blood).

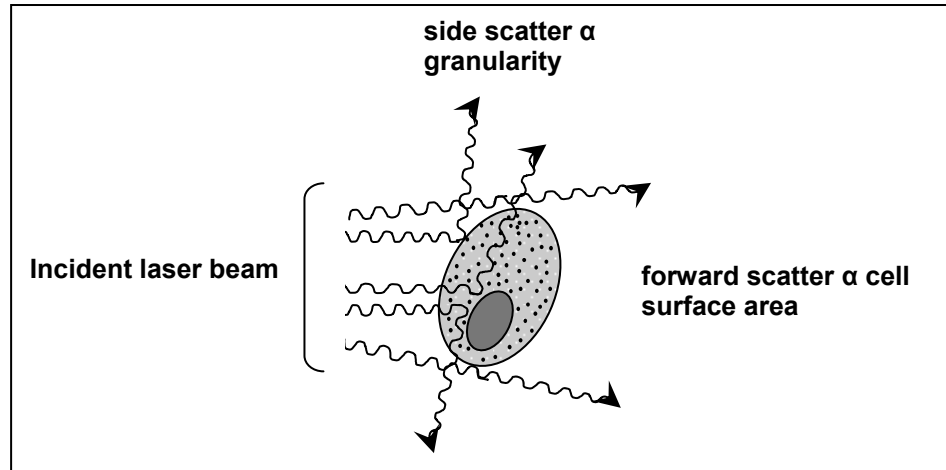


Figure 2-3. Principles of forward scatter and Side scatter. Forward scatter is diffracted light detected along the axis of incident light which is related to cell surface area and thus cell size. Side scatter includes reflected and refracted light which is detected at 90° to the incident beam and is related to cell complexity and granularity. Adapted from ‘introduction to flow cytometry’ from Becton Dickinson online accessed 21st March 2007. http://www.bdbiosciences.com/immunocytometry_systems/support/training/online/ITF/start.html

The main use of flow cytometry is in the analysis of fluorescence. Types of fluorescence measured include autofluorescence, fluorescence of a fluorescently tagged protein (e.g. proteins tagged with Green Fluorescent Protein), fluorescence of a chemical bound to a component of the cell (e.g. propidium iodide or 7-aminoactinomycin-binding to DNA to allow analysis of the cell cycle) or fluorescence of a fluorochrome-linked antibody bound to its specific antigen. In this study fluorochrome labelled antibodies were used for molecular analysis of cell surface expression of the receptor tyrosine kinase, Flk-1. Figure 2-4 illustrates the mechanism of detection of fluorescent molecules.

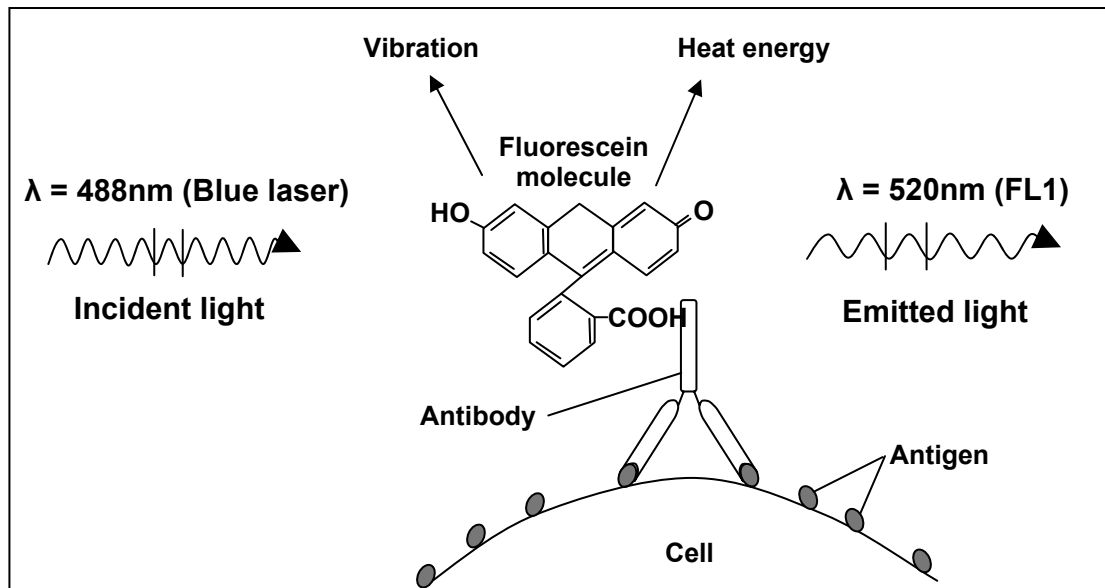


Figure 2-4 Mechanism of fluorescence analysis in flow cytometry. The fluorochrome absorbs the energy of the laser and releases it as heat, vibration and photons of a longer wavelength (in the case of fluorescein 520nm) which is detected by a photomultiplier tube. Adapted from 'introduction to flow cytometry from Beckton Dickinson online accessed 21st March 2007. http://www.bdbiosciences.com/immunocytometry_systems/support/training/online/ITF/start.html

Emitted fluorescence from each cell is converted from a detector current pulse to a voltage pulse which is converted to a log value proportional to its intensity. These values can be displayed in a number of ways, most frequently as either a dot plot or contour plot displaying two parameters against each other (e.g. forward scatter vs. side scatter) or as a histogram plot in which one parameter is plotted against its frequency (e.g. fluorescence). In the case of fluorochrome-labelled antibodies the fluorescence intensity is proportional to the degree of antigen expression. To control for non-specific binding of antibody to the cells an isotype matched control antibody with no specificity for the cell type under study is incubated separately.

In addition to the analysis of cell populations flow cytometry can also be used to sort populations based on their physical characteristics or fluorescence. The target population is selected using computer software and cells of interest are then given a charge. When they pass through positively and negatively charged deflector plates they are deflected out of the stream allowing collection and further analysis

2.4.4.2 Flow cytometry methods used in study

2.4.4.2.1 Analysis of Flk-1 expression

Embryoid bodies or blasts were dissociated using the same protocol as for secondary blast formation. Approximately 0.5×10^6 cells per sample were aliquotted into 1.5ml eppendorf tubes and washed twice in cold FACS block (5% (v/v) FCS, 0.02% (w/v) sodium azide in PBS) (30s at low speed in a refrigerated micro centrifuge) before being re-suspended in 100 μ l of cold FACS block and incubated with 1 μ g/ml of blocking antibody (α -mouseCD16/CD32 Fc γ III/II receptor) for 15 minutes on ice. 5 μ g/ml of primary antibody (α -mouseFlk-1) or isotype control antibody (rat IgG2 κ) was then added and cells were incubated on ice for 30-60 minutes. The cells were then washed twice in 1ml FACS buffer and re-suspended in 100 μ l FACS buffer with 1 μ l of the secondary antibody, (fluorescein isothiocyanate) FITC labelled rabbit- α -rat and incubated on ice for a further 30 minutes. The samples were washed three times, re-suspended in 500 μ l of FACS buffer and transferred to FACS tubes. 1 μ g/ml of the vital dye 7-Aminoactinomycin-D (7AAD) was added to each tube. Live cells were gated on FL3 and 10000 live events were counted to look for an FL1 shift between control and Flk-1 stained cells. FACS was performed on the FACSVantage and FACSCanto and analysis was performed using CellQuest.

2.4.4.2.2 Analysis of apoptosis using DiOC₆ staining

DiOC₆ is a vital dye which specifically stains mitochondria in a membrane integrity dependent manner. Loss of mitochondrial membrane potential is an early irreversible event in apoptosis therefore loss of DiOC₆ staining is indicative of apoptosis (Rottenberg and Wu, 1998).

Cells were dissociated using trypsin and washed in PBS. The cells were resuspended in 1ml PBS and 2 μ l of a 50 μ M DiOC₆ (made up in DMSO) was added. The cells were incubated in the dark at room temperature for 30 minutes, washed once and then subjected to analysis using the Becton Dickinson FACS Vantage for fluorescence on parameter FL1.

Table 2-7 Biochemical and functional techniques consumables

Product	Supplier	Cat. Number
Cell stimulations and preparation of cell extracts		
Aprotinin	Roche Biochemicals, Burgess Hill, UK.	236624
Bromophenol blue	BD electran	44305
Bradford reagent	Bio-Rad	500-0006
EDTA	Sigma	E5134
Glasgow's Modified Eagle Medium	Invitrogen	21710-025
200mM L-Glutamine	Invitrogen	25030-024
Glycerol	Sigma	G5150
1M Hepes	Invitrogen	15630-056
Leupeptin	Sigma	L8511
2-mercaptoethanol	Bio-Rad	161-0710
100X NEAA	Invitrogen	11140-050
Nonidet P40	VWR, Leicestershire, UK.	560092-L
Pepstatin	Sigma	P5318
PMSF	Sigma	H0891
Protein Sepharose A	Amersham/GE Healthcare, Buckinghamshire, UK.	17-0780-01
Protein Sepharose G	Amersham/GE Healthcare	17-0618-01
Sodium Chloride	Sigma	S7653
SDS	VWR	442444-H
Sodium Fluoride	Sigma	S6521
Sodium Molybdate	VWR	102542-Q
Sodium Vanadate	Sigma	S6508
Soybean Trypsin inhibitor	Sigma	T9003
Trizma base	Sigma	T8404
mVEGF	Peprtech	450-32B
Protein resolution and immunoblotting		
30% Acrylamide/Bis 37.5:1	Bio-Rad	161-0158
Ammonium persuphate	Sigma	A7460
Antibodies		
4G10 anti-pY	UBI, USA	05-321
α -phospho-ERK1/2	Cell Signaling Technologies, Hertfordshire, UK.	9101S
α - ERK1/2	Santa Cruz	sc-93
α -phospho- Flk-1* (Tyr 1175) (19A10)	Cell Signaling	2478
α - Flk-1* (55B11)	Cell Signaling	2479

α -phospho-GSK-3 α/β	Cell Signaling	9331
α - GSK-3 β	Cell Signaling	9315
α -p85 (PI3K)	UBI	06-195
α -phospho-PKB/Akt	Cell Signaling	9271
α - PKB/Akt	Cell Signaling	9272
α -phospho- PKB/Akt substrate	Cell Signalling	9611
α -phospho-PKC (pan)	Cell Signaling	9379
α -SHP-2 (C18)	Santa Cruz, UK	sc-280
α -phospho-S6	Cell Signaling	2211
Bovine Serum Albumen (BSA)	Roche Biochemicals	735-108
ECL	Amersham/GE Healthcare	RPN-2106
ECL advance	Amersham/GE Healthcare	RPN-2135
Gel loading tips	GBO	770280
Glycine	Sigma	G8790
Methanol	Fisher	M-4056-17
2-mercaptoethanol	Bio-Rad	161-0710
Nitrocellulose	VWR	43610-6D
Ovalbumen	Sigma	A5378
Ponceau S	Sigma	P7170
Sodium azide	Fisher	S-2380-48
Sodium Chloride	Sigma	S7653
SDS	VWR	442444-H
TEMED	Sigma	T9281
96 well tissue culture plate – flat bottomed	GBO	655-180
Trizma base	Sigma	T8404
3MM Whatman paper	VWR	3030917
Flow cytometry		
7AAD	Sigma	A9400
DiOC ₆	Calbiochem	305110
FACs tubes	BD Biosciences	6297461
FITC-conjugated rabbit α -rat	Dako cytomation systems, Cambridgeshire, UK.	F0234
Phosphate Buffered Saline (PBS)	Invitrogen	18912-014
Sodium azide	Fisher	S-2380-48
Rat α -mouse CD16/CD32 (Fc γ III/II receptor) (Mouse Fc block)	BD Pharmingen, Oxford, UK.	553142
Rat α -IgG _{2α} κ (isotype control)	BD Pharmingen	553927
Rat α -mouse Flk-1	BD Pharmingen	555307

2.5 Molecular Techniques

2.5.1 Expansion and purification of plasmid DNA

2.5.1.1 Preparation of competent Eschericia Coli strain DH5α

A glycerol stock of DH5α was streaked out onto a fresh 2YT agar plate (16g bacto-tryptone, 10g yeast extract, 10g NaCl, 15g bacto-agar made up to 1l and autoclaved) using good aseptic technique and grown inverted overnight at 37°C. A colony off the plate was inoculated into 5ml of 2YT medium (without bacto-agar) and incubated overnight at 37 °C with moderate agitation (~250 r.p.m). The following day the culture was diluted 1:100 in 100ml 2YT broth and grown at 37 °C with moderate agitation until an Optical Density of 0.48 was reached. The culture was then chilled on ice for 5 minutes before centrifugation for 5 minutes at 1380g (3000r.p.m) in a Beckman RC5B with a JA14 rotor at 4°C. The bacterial pellet was gently resuspended in 40ml of solution TfbI (30mM KCl, 100mM RbCl, 10mM CaCl₂, 50mM MnCl₂ and 15% (v/v) glycerol adjusted to pH 5.8 with 0.2M acetic acid), chilled on ice for 5 minutes and centrifuged at 1380g for 5 minutes at 4°C. The supernatant was carefully removed and the smeared pellet was resuspended in 4mls of TfbII solution (10mM MOPS, 75mM CaCl₂, 10mM RbCl, 15% (v/v) glycerol adjusted to pH 6.5 with KOH) and incubated on ice for 15 minutes. Working aliquots were snap frozen in a dry-ice/ethanol bath and stored at -80°C.

2.5.1.2 Transformation of competent E.Coli

Competent bacteria were thawed at room temperature and chilled on ice for 10 minutes. Plasmid DNA was added at a concentration of <100ng/200µl cells, mixed gently and left on ice for 30 minutes. The bacteria were then heat shocked at 42°C for 90s and returned to ice for 2 minutes. 4 volumes of Luria-Bertani (LB) broth (10g bacto-tryptone, 5g yeast extract, 5g NaCl made up to 1l and autoclaved) were added and the bacteria incubated at 37°C for 45 minutes. The required amount of bacteria (depending on expected transformation efficiency) were then plated out onto 2YT agar plates containing an appropriate antibiotic (ampicillin at 100µg/ml or kanamycin at 50 µg/ml).

2.5.1.3 Small-scale plasmid preparation (mini-preps)

A single bacterial colony was picked and grown up in 3ml of 2YT or LB, with addition of an appropriate antibiotic, overnight, at 37°C with vigorous shaking. 1ml of culture was removed to an eppendorf and pelleted by centrifugation at full speed for 1 minute in a microcentrifuge.

The DNA was then extracted using either the Sigma Genelute plasmid mini-prep kit, following the manufacturer's protocol, or by an alkaline lysis protocol.

Alkaline lysis protocol

The cell pellet was lysed in 100µl of solution I (50mM glucose, 10mM EDTA, 25mM Tris-HCl pH8.0). The chromosomal DNA and cell debris were then precipitated in 200µl of fresh solution II (0.2M NaOH, 1% (w/v) SDS) and 150µl of ice cold solution III (3M potassium acetate, 11.5% glacial acetic acid). The cell debris were pelleted at full speed in a refrigerated microcentrifuge at 4°C and the plasmid DNA extracted using phenol:chloroform extraction and ethanol precipitation. (2.4.1.5 and 2.4.1.6)

Sigma Genelute protocol

The cell pellet was resuspended in 200µl of resuspension solution containing RNase and lysed by addition of 200µl of lysis solution with gentle inversion. This solution was left to clear for 5 minutes then neutralised by addition of 350µl of neutralisation solution which caused precipitation of chromosomal DNA and cell debris. The cell debris was pelleted by centrifugation at full speed in a microcentrifuge. The cleared lysate containing the plasmid DNA was transferred to the pre-equilibrated binding column and centrifuged for 30s at $\geq 12000g$. The column was then washed in optional wash solution (medium salt buffer) followed by wash solution (70% ethanol) to ensure removal of contaminants before being eluted in 50-100µl of autoclaved milliQ water and stored at - 20°C.

2.5.1.4 Large scale plasmid preparation (midi or maxi prep)

The Qiagen midi-prep and maxi-prep kits were used following the manufacturers protocols to purify large amounts of ultra-pure supercoiled plasmid for use in applications such as transfection and sequencing. Briefly, a fresh bacterial culture was grown overnight at 37°C with vigorous shaking in LB or 2YT with appropriate antibiotic selection. Bacteria were harvested by centrifugation at 6000 x g for 15

minutes at 4°C in a Sorvall RC5B centrifuge with a JA14 rotor. Plasmid purification was performed using a modified alkaline lysis protocol followed by binding of plasmid DNA under low salt and appropriate pH conditions to a Qiagen anion exchange resin column. Impurities such as RNA and protein were removed in a medium salt wash. A high salt buffer was used to elute plasmid DNA which was then concentrated and desalted in an isopropanol precipitation, resuspended in autoclaved milliQ water and stored at -20°C.

2.5.1.5 Phenol chloroform extraction

DNA in solution was extracted with an equal volume of buffer saturated phenol:chloroform (1:1) and centrifuged for 1 minute at full speed (~20000g) in a microcentrifuge. The upper aqueous phase was removed into a fresh tube and extracted with an equal volume of chloroform. This was again centrifuged at full speed for 1 minute and the upper aqueous phase was again removed into a fresh tube. DNA was then extracted by ethanol precipitation.

2.5.1.6 Ethanol precipitation

0.1 volume of 3M sodium acetate and 2 volumes of 100% ethanol were added to the DNA containing solution and mixed thoroughly. The tube was incubated in a dry-ice ethanol bath until frozen then thawed briefly and spun at full speed (~20000g) in a microcentrifuge for 10 minutes. The ethanol was aspirated and the pellet washed with 70% ethanol. This ethanol was aspirated (in a class 2 tissue culture hood if it was to be used for transfections) and the pellet was left to dry. Once dry the pellet was resuspended in an appropriate volume of autoclaved Tris-EDTA (TE) buffer (10mM Tris-HCl pH 8.0, 0.1M EDTA pH 8.0) or autoclaved milli-Q water and stored at -20°C.

2.5.1.7 Quantification of DNA

DNA was quantified using a GeneQuant II spectrophotometer which measured absorbance at 260nm and 280nm and calculated DNA concentration as follows:

$$\text{Concentration of DNA } (\mu\text{g/ml}) = \text{Dilution factor} \times 50^* \times A_{260}$$

* The standard consideration that 1.0 A_{260} dsDNA = 50 $\mu\text{g/ml}$

The A_{260}/A_{280} ratio can be used as a guide to DNA purity. A ratio of >1.8 indicates little protein contamination. However, this ratio is pH sensitive and can often be unreliable if water is used as the diluent.

2.5.2 Manipulation and analysis of DNA

2.5.2.1 Restriction enzyme digest

Restriction enzyme digests were performed to linearise plasmids, remove a target sequence from a plasmid, create compatible cohesive ends for ligation or as a diagnostic tool for analysis of ligation products. Digests were performed using restriction enzymes supplied by New England Biolabs provided with the appropriate 10x buffer and 100x BSA (10mg/ml) if required. The standard restriction enzyme digest was carried out in 25 μ l and contained 1-5 μ g of DNA, 2.5 μ l of 10X buffer and 2.5 μ l of 1mg/ml BSA (when necessary) made up to 24 μ l with autoclaved milli-Q water, to which 1 μ l of restriction enzyme was added. The digest was then incubated at 37°C for 1-16h. Total reaction volume, amounts of DNA and restriction enzyme and incubation temperature and duration varied depending upon the utility of the digest, the star activity of the enzyme and the activity of the enzyme being utilised. Reactions were stopped where possible by heat activation, or by phenol chloroform extraction. Certain restriction enzymes were used together in double-digests. In these cases a chart provided by NEB advised of the most suitable buffer/BSA combination. Some restriction enzymes were active in PCR buffer allowing removal of a purification step during cloning.

2.5.2.2 Ligation

Ligation is the joining of linear DNA fragments by formation of phosphodiester bonds between 5' and 3' hydroxyl termini in duplex DNA catalysed by T4 DNA ligase. Prior to ligation both vector and insert were digested by restriction enzymes to create compatible 'sticky' or 'blunt' ends (2.4.2.1). When a single restriction enzyme had been used to cut the vector it was then treated with 1 μ l of calf intestinal alkaline phosphatase for 10 minutes at 37°C to remove the 5' phosphoryl termini and prevent self-ligation of the vector. This reaction was stopped by addition of 1 μ l of 0.5M pH8.0 EDTA. Both insert and vector were gel purified (2.4.2.4) and quantified relatively by running on an agarose gel (2.4.2.3), or absolutely via spectrophotometry

(2.4.1.7). Ligations were carried out in 20µl volumes containing 2µl 10x T4 DNA ligase buffer (50mM Tris-HCl, 10mM MgCl₂, 10mM DTT, 1mM ATP, 25µg/ml BSA, pH7.5), 100ng of vector, insert, autoclaved milliQ to 18µl and 2µl of T4 DNA ligase. Ligations were incubated at 16°C overnight. Sticky end ligations were performed using a 2:1 molar ratio of insert to vector for small inserts and a 4:1 molar ratio for larger inserts. Blunt ended ligations were performed with a large excess of insert over vector. Absolute amounts of DNA required were calculated using the following formula:

$$\text{Insert (ng)} = N \times [(\text{vector (ng)} \times \text{size of insert (kb)}) / \text{size of vector (kb)}]$$

Where N = molar ratio of insert to vector

Control ligations in which ligase, vector or insert were lacking were also performed.

2.5.2.3 Agarose gel electrophoresis

Agarose gels were used in the analysis of DNA and the purification of DNA fragments. An appropriate quantity of agarose (see table 2-7) was dissolved by boiling in Tris-acetate EDTA (TAE) buffer (50 x stock; 242g Tris, 57.1ml glacial acetic acid, 100ml 0.5M EDTA pH 8.0). Once cooled, the agarose gel was poured into pre-assembled agarose gel tank and a well forming comb was inserted. The set gel was placed in an electrophoresis chamber and samples made up in DNA loading buffer (6 x loading buffer; 30% (v/v) glycerol, 0.05% (w/v) bromophenol blue) were loaded. Electrophoresis was performed at 70-90V for the desired time. The gel was stained for 5 minutes in ~0.5µg/ml ethidium bromide in TAE and visualised and photographed using a Syngene UV transilluminator and Genesnap software.

Table 2-8 Resolving power of differing agarose percentages

% gel	Optimum Resolution for Linear DNA (kb)
0.5	30 to 1.0
0.7	12 – 0.8
1.0	10 to 0.5
1.2	7 – 0.4
1.5	3 – 0.2
2.0	1.5 – 0.05

Table 2-9 Plasmids used in this study (maps in Appendix)

Plasmid	Derivation	Use
pcDNA3.1Zeo (Invitrogen)	Mammalian expression plasmid containing the CMV early immediate promoter and Ampicillin and Zeocin resistance genes.	Tractable vector used for initial cloning of Flk-1.
pcDNA3.1Zeo- <i>Ms</i> Flk-1	The Murine Flk-1 sequence ligated into pcDNA3.1zeo using the XbaI restriction site	Used to check viability of cloned Flk-1 sequence in BaF/3 cell line and for sequencing.
pCAGGS-irtTA-VP16-IRES-puro (Anastassiadis et al., 2002)	Reverse Tetracycline transactivator with puromycin resistance gene.	Enables regulated expression by addition of doxycycline of genes expressed in the tet response plasmid.
pKS-TetTK-pA (Anastassiadis et al., 2002)	Bluescript vector modified to encode tet operators, a thymidine kinase (TK) promoter and polyA tail.	Used for insertion of Flk-1 sequence into TetTK-pA.
pKS-TetTK-flk-1-pA	Flk-1 ligated into pKS-TetTK-pA using the SpeI restriction site	Used to insert TetTK-Flk-1-pA sequence into Inshygro vector.
pKS- Δ p85-TetTK-pA	Myc tagged p85 subunit lacking the binding site for the catalytically active p110 component of PI3K ligated into pKS-TetTK-pA.	To determine the presence of functional tetracycline transactivator in stably transfected R1 ES cells using transient tranfection.
pAc Δ PHAkt-GFP-N1	pACGFP-N1 vector (Clontech) expressing GFP tagged Akt PH domain non-lipid binding mutant	Optimising transient transfection of R1 ES cells using flow cytometry to detect GFP expression
pInshygro (Anastassiadis et al., 2002)	Expression vector containing 4 chicken β -globin chromatin insulators flanking the multiple cloning site and a hygromycin resistance gene.	Insulators thought to block inappropriate action of regulatory elements reducing likelihood of mosaic expression. Hygromycin resistance enables selection.
pInshygro-TetTK-Flk-1-pA	TetTKFlk-1-pA blunt end ligated into the multiple cloning site of the SnaBI digested Inshygro plasmid	Construct linearised and transfected into Flk-1 ^{-/-} R1 ES cells stably expressing CAGGS-irtTA-VP16-IRES-puro

2.5.2.4 Gel purification

DNA fragments excised from agarose gels under UV were purified using a Qiaquick gel extraction kit. Briefly, the gel was solubilised at 50°C buffer QG, loaded onto a Qiaquick silica column and spun down. The flow through was discarded and the column washed once with buffer QG and once with buffer PE, an ethanol containing wash buffer. DNA was eluted in 30-50µl of autoclaved milli-Q water.

2.5.2.5 Sequencing

Mini-prepped plasmid DNA and sequencing primers were sent to the sequencing facility in the South Building, University of Bath or the sequencing facility in the Biochemical department at Oxford University. Sequence analysis was performed using the Chromas and Sequencher programs.

2.5.3 Cloning and expression analysis

2.5.3.1 RNA extraction

RNA extraction was performed for either relative expression analysis or cloning of genes of interest. $1-10 \times 10^6$ cells were lysed in 1ml of Trizol in a 1.5ml eppendorf tube and incubated for 5 minutes at room temperature. For embryoid bodies an 18G blunt needle on a 2ml syringe was used to aid homogenisation. 200µl of chloroform was added and the tube was vigorously shaken for 15s. After 2-3 minutes incubation at room temperature to allow separation of the two phases centrifugation was performed at full speed (~20000g) for 15 minutes at 4°C. The upper aqueous phase was transferred to a sterile eppendorf and the RNA was precipitated by addition of 0.5ml of isopropanol and incubation on ice for 10 minutes. This was followed by a 10 minute full speed centrifugation step at 4°C to pellet the precipitated RNA. The RNA pellet was washed in 70% ethanol and centrifuged for 5 minutes at 4 °C. The ethanol was aspirated and the pellet left to air dry before being resuspended in 20-100µl of autoclaved milli-Q water and incubated for 10 minutes at 55°C to denature secondary structure and aid solubilisation of RNA. RNA was stored at -80°C.

2.5.3.2 RNA quantification

RNA was quantified using the GeneQuant II following the same protocol for DNA quantification according to the standard consideration;

$$1.0 A_{260} \text{ RNA} = 40\mu\text{g/ml}$$

If RNA integrity was in question it was checked by running an aliquot of RNA on an agarose gel using gel electrophoresis equipment rendered RNase free by washing in 3% H₂O₂. Non-degraded RNA runs in two sharp bands 28s and 18s with the 28s band being approximately twice as intense in non-degraded RNA.

2.5.3.3 Reverse Transcription- Polymerase Chain Reaction (RT-PCR)

RT-PCR utilises reverse transcriptase to synthesise cDNA for amplification in the polymerase chain reaction (PCR) from mRNA of expressed genes.

When RT-PCR was used to produce cDNA from which a full length clone would be amplified 50ng of random primers and 1mM dNTPs were mixed with 2µg of RNA in 17µl total volume, incubated at 65°C for 5 minutes then immediately chilled on ice for 2 minutes. 4µl of first strand sample buffer, 1µl of RNasin and 5mM DTT were then added and the reaction was mixed well and incubated at room temperature for 2 minutes. 1µl (200 units) of superscript II or superscript III reverse transcriptase was then added. A duplicate reaction mix containing no reverse transcriptase was included for each sample to control for expansion of contaminating genomic DNA contamination. Reverse transcription took place as follows in a Techne Touchgene gradient PCR machine:

25° C	10 minutes
42°C (50°C for Superscript III)	50 minutes
70°C	15 minutes
4 °C	hold

1µl of Ribonuclease H was added to each reaction and incubated at 37°C for 20 minutes to specifically degrade the RNA strand of any RNA:DNA hybrids.

When RT-PCR was used to produce cDNA for expression analysis it was first DNase treated prior to RT-PCR to remove any contaminating genomic DNA. 1µg of RNA was incubated in an 11µl reaction volume with 1µl of 10X DNase buffer and 1µl of DNase at 37°C for 30 minutes. The enzyme was denatured by addition of 1µl of

DNase stop solution (0.5M pH8.0 EDTA) and incubation at 65° for 10 minutes. 0.5ng of oligo-dT was added, mixed well and incubated at 65°C for 5 minutes. 4µl of first strand sample buffer, 1µl of RNasin, 5mM DTT and 500µM dNTPs were then added and the reaction was mixed well. Finally 1µl of superscript II was added and reverse transcription was performed at 42°C for 50 minutes followed by a 15 minutes 70°C inactivation step in a Techne Touchgene gradient PCR machine. Duplicate minus reverse transcriptase reactions were also performed and cDNA was stored at -20°C.

2.5.3.4 Polymerase Chain Reaction (PCR)

PCR is an enzymatic method for replicating DNA allowing a small amount of DNA to be amplified exponentially.

For amplification of cDNA for expression analysis the *Thermus aquaticus*- *Taq* DNA polymerase which has a high amplification rate but low proof reading activity was used. 1µl of cDNA from a reverse transcription reaction was amplified in Magnesium free buffer (10mM Tris-HCl pH 9.0, 50mM KCl, 0.1% Triton X-100) containing 250µM dNTPs, 5pmol of both sense and antisense primers, 2mM MgCl₂ and 0.1µl *Taq* in a 20µl reaction under the following conditions in a Techne Touchgene gradient PCR machine;

Initial denaturation	94 °C for 5 minutes
20-40 cycles Denaturation	94°C for 30s
Annealing*	X°C for 30s
Elongation	72°C for 1 minute
Final elongation	72°C for 5 minutes
	4°C hold

For amplification of cDNA for cloning a proof reading polymerase with higher fidelity but a lower amplification rate was used. The *Pyrococcus furiosus* *Pfu* proof reading polymerase was used most commonly though *Pfx*, *accuase* and *KOD* hot start proof reading enzymes were also used. 5µl of cDNA from a reverse transcription reaction was amplified in *Pfu* polymerase reaction buffer (20mM Tris-HCl pH 8.8, 10mM KCl, 10mM (NH₄)₂SO₄, 2mM MgSO₄, 0.1% Triton X-100,

0.1mg/ml nuclease free BSA) containing 200µM dNTPs, 12.5pmol of both sense and antisense primers and 1µl of *Pfu* in a 50µl reaction volume under the following conditions in a Techne Touchgene gradient PCR machine;

Initial denaturation	94°C for 5 minutes
40 cycles	Denaturation 94°C for 30s
	Annealling* X°C for 30s
	Extension 72°C for 2 minutes/kb to be amplified
Final extension	72°C for 15 minutes
	4°C hold

* Annealling temperature was determined empirically for each primer set based upon the melting temperature of the primers (2.4.3.5).

2.5.3.5 *Primer design*

In designing primers, where ever possible the following rules of primer design were adhered to; (Innis MA and Gelfand DH, 1990)

1. Primers should be between 17 and 28 base pairs in length.
2. Base composition of 50-60% GC is optimal.
3. Primers should have a 3' terminal G, C GC or CG 'clamp'.
4. Melting temperature of primers should be in the range 55-80°C.
5. 3' complementarity should be avoided.
6. Internal self complementarity should be avoided.
7. Runs of 3 or more Gs or Cs near the 3' end of primers should be avoided.

Melting temperature or T_m of primers was calculated using the Wallace formula

$$T_m = 4 \times (G+C) + 2 \times (A + T)$$

Primers were checked for formation of dimers or secondary structures such as hairpins using net primer (<http://www.premierbiosoft.com/netprimer/>). Primers used in this study are detailed in Table 2-10

Table 2-10 Primers used in this study

Name	Sequence	Annealing temp (°C)	Use	Source
Flk-1 FL S	TTTTCTAGAATGGAGAGCAAG GCGCTGCT	62	Cloning of full length Flk-1 cDNA incorporating a 5' XbaI restriction site	Designed for this study
Flk-1 FL AS	TTTTCTAGATTAAACAGGAGGT GAGCGCAG	62		
GAPDH S	TGGAGCCAAACGGGTCATC	59		
GAPDH AS	GCTCTGGGATGACCTTGCC	59	Used as controls in expression analysis	C Beck, pers. comm., M.Storm, pers. comm.
β-actin S	TAGGCACCAGGGTGTGATGG	62		
β-actin AS	CATGGCTGGGGTGTGAAGG	62		
β-H1 S	AGTCCCCATGGAGTCAAAGA	56	Expression analysis	Faloon <i>et al.</i> , 2000
β-H1 AS	CTCAAGGAGACCTTTGCTCA	56		
β-major S	CTGACAGATGCTCTCTTGGG	56		
β-major AS	CACAACCCAGAAACAGACA	56		
Brachyury S	CATGTACTCTTCTTGCTGG	58		
Brachyury AS	GGTCTCGGGAAAGCAGTGGC	58		
c-kit S	TGTCTCTCCAGTTTCCCTGC	60		
c-kit AS	TTCAGGGACTCATGGGCTCA	60	Expression analysis and sequencing	Keller <i>et al.</i> , 1993
Flk-1 S	CACCTGGCACTCTCCACCTTC	60		
Flk-1 AS	GATTTTCATCCCACTACCGAAAG	60		
Scl S	ATTGCACACACACGGGATTCTG	56	Expression analysis	Faloon <i>et al.</i> , 2000
Scl AS	GAATTCAGGGTCTTCCTTAG	56		
VEGF S	CCAAAGCCAGCACATAGG	54	Sequencing	Designed
VEGF AS	GGTGACATGGTTAATCGG	54		
1688 SEQ	CCCGTATGCTTGTAAGAATGG			
2284 SEQ	CAACAACCATTGGCGAGA			
3600 SEQ	GGTAGTGTAGTCAGGAGCCCG			
OUTSIDE	TTGCTAACTAGAGAACCCACTG			
REV SEQ	C			

Table 2-11 Molecular techniques consumables

Product	Supplier	Cat. Number
Expansion and purification of plamid DNA		
Acetic acid	Sigma	537020
Agar	BD Difco	281230
Ampicillin	Sigma	A9518
Bacto-tryptone	Sigma	T2559
Bromophenol blue	BD electran	44305
Buffer saturated phenol	Invitrogen	13513-039
Calcium chloride	Sigma	C5080
Chloroform	Fisher	C-4960-17
EDTA	Fisher	S75203
Glucose	Sigma	G5400
Glycerol	Sigma	G5150
Kanamycin	Sigma	K4000
Manganese chloride	Sigma	M3634
MOPS	Sigma	M5162
Potassium acetate	Sigma	P1190
Potassium chloride	Sigma	P9591
Qiagen midi prep kit	Qiagen, Crawley, West Sussex, UK.	12143
Qiagen maxi prep kit	Qiagen	12362
Rubidium Chloride	Sigma	R2252
Sigma Genelute plasmid mini prep kit	Sigma	PLN 70
Sodium acetate	Fisher	S75203
Sodium chloride	Sigma	S7653
Trizma Base	Sigma	T8404
Yeast extract	BD Difco	288620
Manipulation and analysis of DNA		
Glacial Acetic acid	Sigma	537020
Agarose	Invitrogen	15510-027
Bromophenol blue	BD electran	44305
CIP alkaline phosphatase	Invitrogen	M0210S
EDTA	Fisher	S75203
Ethidium Bromide	Bio-Rad	1610433
Glycerol	Sigma	G5150
Qiaquick gel extraction kit	Qiagen	28704
Restriction enzymes	New England Biolabs	Various
T4 DNA ligase	Invitrogen	5224017
Trizma Base	Sigma	T8404
Cloning and expression analysis		
Chloroform	Fisher	C-4960-17
DNase	Promega	M6101
dNTPs	Invitrogen	10297-018
<i>Pfu</i> polymerase	Promega	M7741
Primers	Invitrogen	-

RNase free eppendorfs	Eppendorf	3810X
rRNasin	Promega	N2511
Ribonuclease H	Invitrogen	18021-014
Superscript II reverse transcriptase	Invitrogen	18064-014
Superscript III reverse transcriptase	Invitrogen	18080-044
Taq polymerase	Promega	M1661
Trizol	Invitrogen	15596-018

2.6 Preparation and characterisation of transfectants

2.6.1 Transfection

2.6.1.1 Stable transfection of BaF/3 cells

BaF/3 cells were washed 3 times in filter sterilised electroporation buffer (25mM HEPES, pH7.2, 140mM KCl, 10mM NaCl, 2mM MgCl₂, 0.5% Ficoll 400) then resuspended at 2.5×10^7 /ml in 0.8ml of electroporation buffer in an electroporation cuvette containing 10µg of linearised plasmid DNA.

Electroporation was performed at 960µF, 450V. Cells were transferred into 20ml of complete BaF/3 media for 48 hours after which they were plated at 5×10^4 - 2×10^5 cells/well of a 96 well plate and supplemented with 600µg/ml of zeocin (Invitrogen) for selection. Fresh media was added after 1 week to maintain antibiotic resistance and resistant clones were then expanded for screening and freezing (see section 5.2.3).

2.6.1.2 Stable transfection of ES cells

R1 Flk-1^{-/-} ES cells were washed three times in sterile PBS w/o Ca²⁺ and Mg²⁺ and resuspended at 1.25×10^7 /ml in 0.8ml of PBS w/o Ca²⁺ and Mg²⁺ in an electroporation cuvette containing 20µg of linearised plasmid DNA. The cuvette was incubated on ice for 5 minutes prior to electroporation at 500µF, 250V (Prelle et al., 2000). After a further 5 minute incubation on ice the cells were plated out at 2×10^6 /10cm dish in ES cell medium supplemented with 1% hyclone FCS. After 24 – 48 hours appropriate selection was added (hygromycin at 50µg/ml or puromycin at

1µg/ml). Cells were washed and media replaced every day until clones were clearly visible (approximately 8 days in selection for hygromycin selection and 5-6 days for puromycin).

When clones were clearly visible plates were gently washed in PBS which was then thoroughly aspirated. Up to 7 clones per plate were picked. Using sterile forceps the smooth end of a cloning ring was coated in autoclaved petroleum jelly and placed over a colony. A small amount of trypsin-EDTA was then applied to dissociate the colony and a 200µl pipette was used to transfer the colony to 1ml of selection media in one well of a 24 well plate. Clones were then expanded for screening and freezing (see section 5.2.3.3)

2.6.1.3 Transient transfection of ES cells

Electroporation was found to significantly decrease the viability of R1 ES cells therefore transient transfection was optimised using lipofectamine 2000 lipid based transfection system (see section 5.2.3.1). 8µg of plasmid DNA was diluted in 1ml of optimem media and mixed with 20µl of lipofectamine 2000 also diluted in 1ml of optimem. Following a 20 minute incubation at room temperature the plasmid:lipofectamine mixture was added to 1×10^6 cells in 10ml of ES cell media. Medium was changed 8 hours later and cells were checked for expression after a further 16-64 hours.

2.6.1.4 Screening clones

To screen Flk-1^{-/-} ES cells transfected with a dox-inducible-Flk-1 construct doxycycline was added at 1µg/ml for 24-48 hours to induce expression. Clones were screened using RNA extraction followed by RT-PCR or by flow cytometry (2.4.3 and 2.3.5 respectively). To screen clones transfected with the tet-regulator plasmid pCAGGS-irtTA-VP16-IRES-puro cells were transiently transfected with a tet-response plasmid prior to screening.

Table 2-12 Transfection consumables

Product	Supplier	Cat. Number
Transfection		
Cloning rings	Made in house by cutting the wide end off a p1000 pipette tip with a hot scalpel blade and autoclaving	
doxycycline	Sigma	D-9891
Electroporation cuvettes	Bio-Rad	1652088
Ficoll 400	Sigma	F-4375
1M Hepes	Invitrogen	15630-056
hygromycin	Calbiochem	400049
Lipofectamine-2000	Invitrogen	11668-019
Magnesium Chloride (MgCl ₂)	Sigma	M-9272
Optimem	Invitrogen	31985-047
Potassium chloride (KCl)	Sigma	P-9541
puromycin	Calbiochem	540222
Sodium Chloride (NaCl)	Sigma	S-7653
zeocin	Invitrogen	R250-01

Chapter 3

Flk-1 expression and signalling in models of early development

3.1 Introduction

The receptor tyrosine kinase Flk-1 plays an important role in haemopoietic and endothelial development in early embryogenesis (Shalaby et al., 1995) marking the population from which the haemangioblast (Blast-Colony Forming Cell (BL-CFC) *in vitro*), the common precursor of haemopoietic and vascular development, arises (Choi et al., 1998; Fehling et al., 2003; Huber et al., 2004) (see section 1.4 for further discussion). Upon differentiation of the haemangioblast Flk-1 expression is lost on haemopoietic precursors, but is maintained on the endothelial lineage into adult life (Kabrun et al., 1997). In the adult Flk-1 mediates angiogenesis, an important target in the pathology of many human diseases including cancer, Rheumatoid Arthritis, ocular neovascularising disease and cardiovascular disease (Zachary, 2001). The signalling mediated by the human homologue of Flk-1, KDR has been well characterised but little is known about the signalling mediated by Flk-1 during early embryogenesis in mouse and man (Zachary, 2001).

Once identified, the signalling pathways activated by Flk-1 during early development could be used to further dissect its developmental role. To evaluate signalling mediated by Flk-1 in early murine development cells dissociated embryoid bodies or blast colonies were utilised. Embryoid bodies were used as a model of early embryogenesis and blast colonies were used to evaluate signalling during haemopoietic and endothelial commitment. The D4T embryoid body derived endothelial cell line (Kennedy et al., 1997) and Human Umbilical Vein Endothelial Cells (HUVEC) (Jaffe et al., 1973) were used as control cell lines. Flk-1 surface expression was evaluated using flow cytometry and populations of cells expressing Flk-1 were stimulated with its ligand VEGF. Activation of specific signals were evaluated in cell lysates using immunoblots. Signalling pathways of particular interest included the MAPK pathway and the PI3K pathway both of which are activated by VEGF in adult endothelial cells and have been reported to play an important role in cell proliferation and survival (Dayanir et al., 2001; Doanes et al., 1999; Gliki et al., 2001).

3.2 Flk-1 expression

The up-regulation of Flk-1 expression marks the appearance of the haemangioblast, or BL-CFC population, in the embryo and embryoid body (Fehling et al., 2003; Huber et al., 2004). Kabrun *et. al.* demonstrated Flk-1 expression on embryoid bodies cultured in methylcellulose from day 3.25 after initial plating with expression peaking at around 50% on day 4 declining to ~ 5% by day 12 (Kabrun et al., 1997). It has since been established that the kinetics of Flk-1 expression vary slightly between embryonic stem cell lines (Chung et al., 2002; Fehling et al., 2003; Kabrun et al., 1997). Initial characterisation of Flk-1 expression in differentiating R1 and E14tg2A ES cells, the ES cells to be used in this study, was therefore required to identify the time points where Flk-1 expression was maximal, such that cells at this stage could be used for the analysis of signalling pathways. The kinetics of Flk-1 expression are also important in defining the narrow temporal window of formation of the BL-CFC. Re-plating of dissociated embryoid bodies containing the BL-CFC population into a secondary blast assay allows BL-CFC expansion and differentiation into blast colonies. Flk-1 reportedly decreases during blast colony expansion (D'Souza et al., 2005). The kinetics of Flk-1 expression and its expression levels relative to those in primary embryoid bodies were characterised to confirm this decrease and to investigate the feasibility of secondary blasts as a tool to dissect Flk-1 signalling during BL-CFC expansion and commitment.

Flk-1 expression was detected with a anti-Flk-1 primary antibody raised against the extracellular domain of murine Flk-1 and a FITC conjugated secondary antibody. Cells were then analysed by Flk-1 expression using flow cytometry. An isotype-matched control antibody was used to control for non-specific binding of the anti-Flk-1 antibody and F_c block was used to block antibody binding to F_c receptors. The vital dye 7-Aminoactinomycin (7AAD) was used to gate for viable cells in flow cytometry experiments. For an overview of flow cytometry see section 2.3.5.1.

3.2.1 Methods of data analysis in flow cytometry

Traditionally measures of fluorescence intensity in flow cytometry are displayed on a logarithmic scale. If a single fluorescent parameter has been measured, data is most commonly presented in the histogram format illustrated in Figure 3-1.

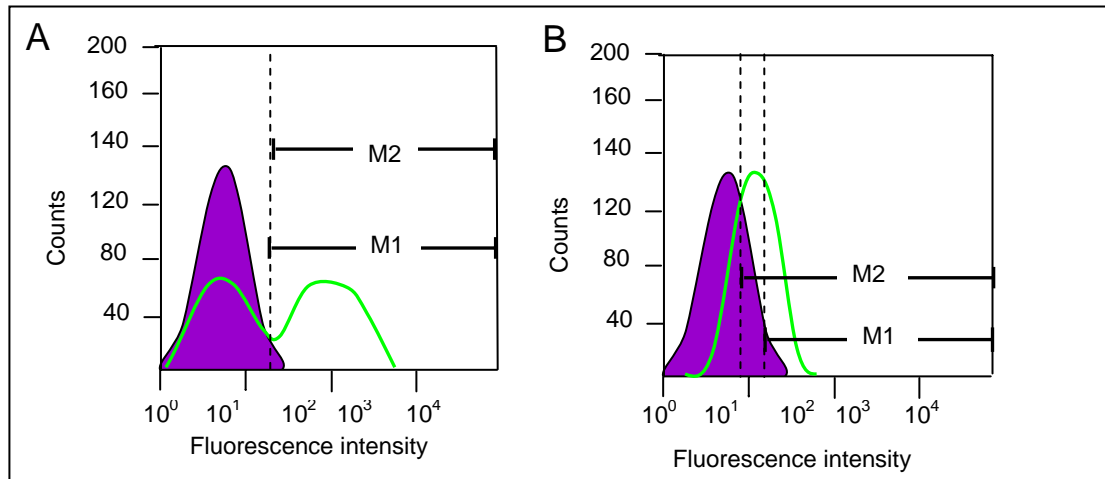


Figure 3-1 Examples of potential histogram overlays. The purple trace represents the isotype or unstained control whilst the green trace represents the antigen specific antibody labelled population. Marker M1 is placed to include 5% of the isotype control peak. Marker M2 is placed at the intercept of isotype control and antigen specific antibody labelled populations. Diagram A show a histogram representative of flow cytometry performed on a mixed population of cells which are either positive or negative for the antigen of interest. Diagram B is representative of a more homogenous population in which the whole population expresses a lower level of antigen.

Data analysis from histogram plots is commonly conducted using markers incorporating a low percentage of the control peak, usually 1% or 5%. The percentage of antigen labelled cells minus the percentage of control cells within the marker is quoted as percentage antigen positive cells. This approach works well for populations with high antigen expression and obvious positive and negative populations such as Figure 3-1A. However, in populations expressing lower antigen levels this approach may exclude some of the cells which appear positive (illustrated by marker M1 in Figure 3-1 B). Placement of the marker at the intercept of control and antigen specific labelled populations (marker M2 in Figure 3-1B) gets round this problem but may yield a falsely high figure. Additionally, marker placement may skew the data. In either case, quoting percentage expression is probably inaccurate as

the shift of the entire population in Figure 3-1B indicates that the whole population is positive but expresses low levels of antigen. Another method of data analysis in such a case is to compare the means or medians of the peaks calculated by the Cellquest FACS analysis software. Geometric mean should be used for comparison of log data to prevent the skewing of the mean by outliers.

3.2.2 Flk-1 expression on cells derived from primary embryoid bodies

To establish a time-course of Flk-1 expression in cells developing in primary embryoid bodies, expression of Flk-1 was measured on populations of embryoid body-derived cells generated from both E14tg2A and R1 ES cell lines between days 3 and 7 of embryoid body formation. This data was compared with that published in the literature (Chung et al., 2002; Fehling et al., 2003; Kabrun et al., 1997).

Histogram plots of a representative time-course for each ES cell line are illustrated in Figure 3-2. In many of the traces (including days 4 and 5 in panels A and B) the α -Flk-1 labelled trace overlaps with the isotype control trace as a single diffuse peak, indicating low overall receptor expression with levels varying within the population.

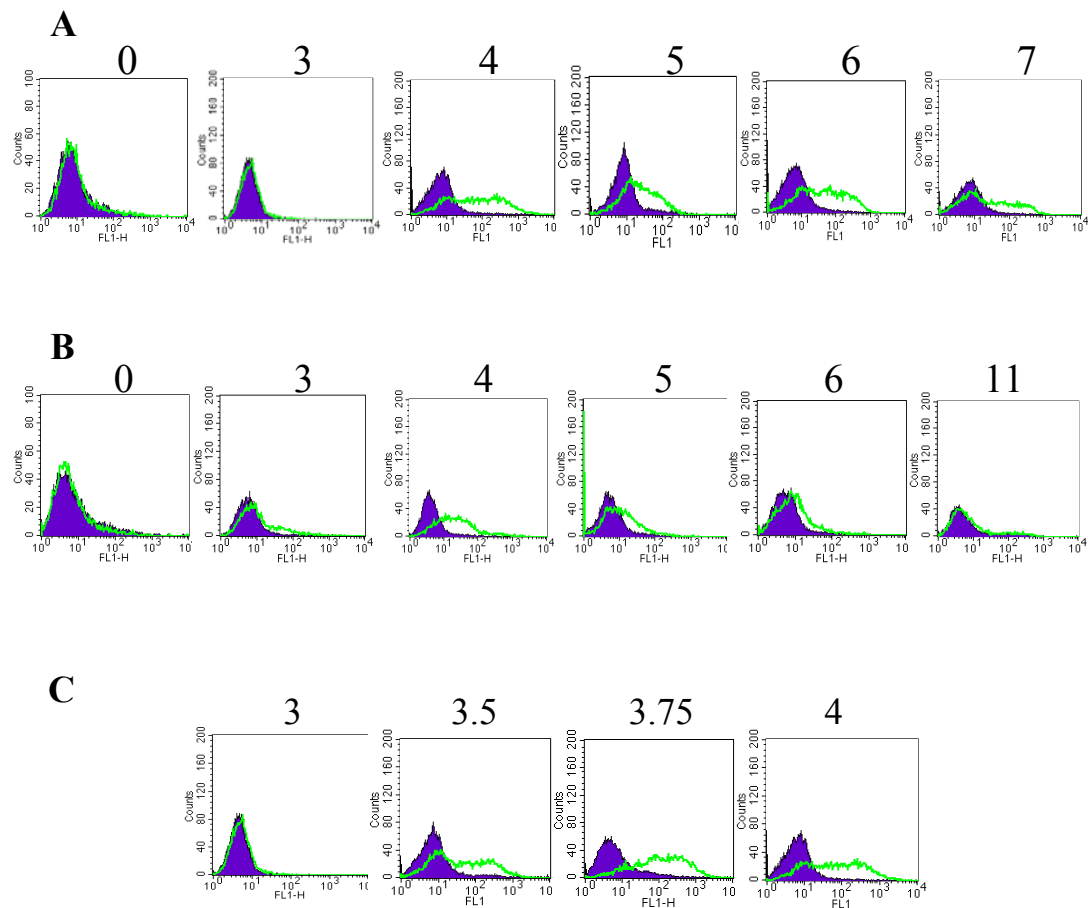


Figure 3-2. Flk-1 expression in cells derived from developing embryoid bodies. Cells from dissociated embryoid bodies that had differentiated for between 3 and 7 days, were stained with an anti-Flk-1 antibody or an equal concentration of isotype matched control and a secondary FITC labelled antibody. The numbers above each plot indicate the number of days the primary embryoid bodies had been allowed to differentiate for. The Y axis represents cell number while the X axis represents fluorescent intensity. The purple peak indicates the isotype control labelled population and the green trace represents the anti-Flk-1 antibody labelled population. **A** illustrates a time-course of Flk-1 expression in cells from dissociated embryoid bodies of the E14tg2A ES cell line. **B** illustrates a time-course of Flk-1 expression in cells from dissociated embryoid bodies of the R1 ES cell line. **C** illustrates fine kinetic analysis of Flk-1 expression in cells from dissociated E14tg2A embryoid bodies. The data in Figure 3-2 A and B is representative of 3 complete time-course experiments with the exception of data from day 11 R1 primary embryoid bodies (n=1). The data represented in Figure 3-2C is representative of 2 complete time-course experiments.

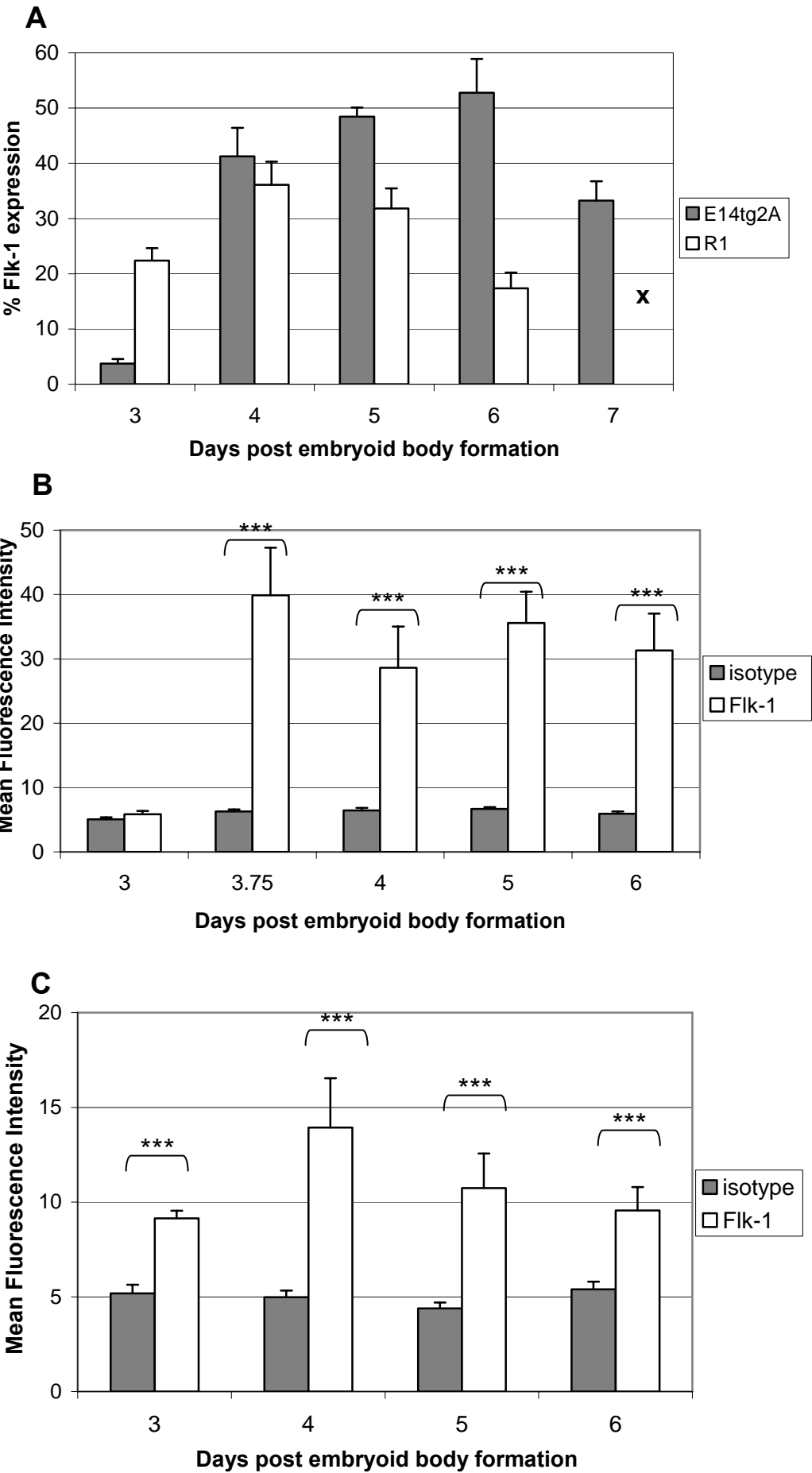


Figure 3-3 Time-course of Flk-1 expression in primary embryoid body-derived cells.

Cells from dissociated embryoid bodies that had differentiated for between 3 and 7 days, were labelled with an anti-Flk-1 antibody or an equal concentration of isotype-matched control and a secondary FITC labelled antibody prior to analysis on a Becton Dickinson FACS Vantage flow cytometer. The percentage of cell expressing Flk-1 and the geometric mean intensity were calculated as described in section 3.2.1. Percentage Flk-1 expression was calculated using a 5% marker gate. The results shown represent pooled data from ≥ 3 individual experiments. **A** Comparison of % Flk-1 expression in cells from dissociated R1 and E14tg2A primary embryoid bodies respectively differentiated for between 3 and 6 days. **B and C** Comparison of the geometric mean fluorescent intensities between isotype control and anti- flk-1 labelled cells from dissociated E14tg2A and R1 embryoid bodies differentiated for between 3 and 6 days. Error bars represent s.e.m, *** $p < 0.01$ in the paired students t-test. Analysis of variance (ANOVA) showed no significant difference in geometric mean fluorescence intensities between anti-Flk-1-labelled embryoid body-derived cells on days 3.75, 4, 5 and 6 in **B** or days 3,4,5 and 6 in **C**. X indicates that no Flk-1 expression data was available.

Figures 3-2A and C and 3-3A and B illustrate the rapid induction of Flk-1 expression between days 3 and 4 of EB development using E14tg2A ES cells. Expression peaked between days 4 and 6 and began to decline thereafter. Flk-1 expression in R1 ES cells, shown in figures 3-2B, 3-3A and C, was induced earlier, by day 3 of embryoid body formation and declined sooner, indicating that differentiation proceeds more rapidly in this cell line. Flk-1 expression peaked at day 4 and declined there after, correlating temporally with a similar time-course of Flk-1 expression in R1 embryoid bodies performed by Chung *et al.*, (Chung et al., 2002).

Both the percentage of Flk-1 expression and geometric mean fluorescence intensities were higher in E14tg2A-derived EBs than in their R1 counterparts indicating that cells within these populations expressed higher levels of receptor. As the E14tg2A time course experiments were not run in tandem with the R1 cell line it is unclear whether this difference is significant. In both ES cell lines the pattern of Flk-1 expression and the expression levels, in terms of percentage of Flk-1 expression, correlate well with data from other workers (Chung et al., 2002; Kabrun et al., 1997). Taking into consideration the possible higher Flk-1 expression levels in the E14tg2A EBs, their expression profile and the cellularity of the embryoid bodies at different

stages, it was decided to perform the signalling experiments using cells from dissociated day 5 E14tg2A embryoid bodies.

3.2.3 Flk-1 expression in secondary blasts

The secondary blast assay is a measure of formation of the transient Blast Colony Forming Cell (BL-CFC) that arises around the time of Flk-1 upregulation (primary embryoid bodies and secondary blasts are illustrated in Figure 2-1). Embryoid bodies containing the BL-CFC population are dissociated and replated into fresh methylcellulose with growth factors including VEGF and D4T conditioned medium to allow expansion and differentiation of the BL-CFC population (Choi et al., 1998; Faloon et al., 2000; Kennedy et al., 1997). The onset of Flk-1 expression marks formation of the BL-CFC. Using the time-course of Flk-1 expression in primary embryoid bodies (Figures 3-2 and 3-3) the window of BL-CFC formation was identified as day 3.5-4 for E14tg2A EBs.

Flk-1 expression is down-regulated upon haemopoietic commitment but maintained on endothelial cells (Kabrun et al., 1997; Nishikawa et al., 1998a; Zachary, 2001) suggesting the receptor should be retained by some of the differentiating BL-CFC population. Northern Blotting by D'Souza *et. al.* demonstrated down-regulation of Flk-1 expression upon blast colony expansion (D'Souza et al., 2005). In order to further investigate the kinetics and levels of Flk-1 expression in secondary blasts flow cytometry was again utilised.

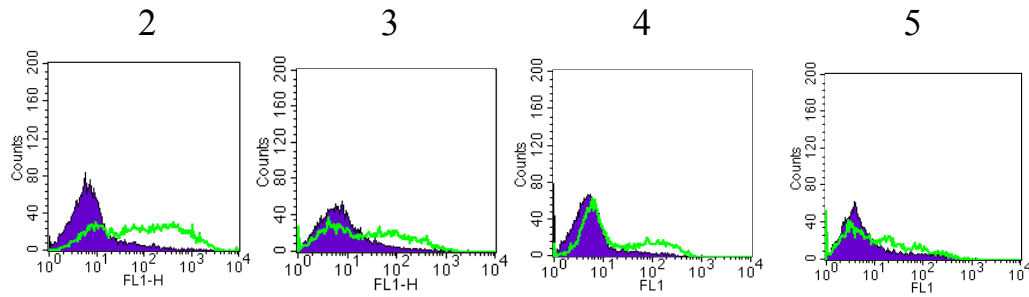


Figure 3-4 Flk-1 expression in dissociated secondary blast cells. Secondary blasts were derived from re-plated dissociated day 3.75 E14tg2A primary embryoid bodies and expanded for between 2 and 5 days. The blast colonies were then dissociated and their constituent cells stained with either an anti-Flk-1 antibody or an equal concentration of isotype-matched control and a secondary FITC labelled antibody. The numbers above the plots indicate the numbers of days of blast colony expansion the cells underwent. The Y axis represents cell number and the X axis represents fluorescent intensity. The purple trace indicates the isotype control population whilst the green trace indicates the anti-Flk-1 antibody stained population. The data shown are representative of 3 complete time-course experiments with the exception of day 5 which is representative of 2 individual experiments.

The data shown in Figure 3-4 demonstrates that in contrast to Flk-1 expression in primary embryoid body-derived cells, Flk-1 expression in secondary blasts appears to separate into two clear populations, Flk-1 positive and Flk-1 negative. The fluorescence intensity of the Flk-1 positive population is also higher than that observed in the primary embryoid bodies (see Figure 3-2), suggesting higher levels of receptor expression on the positive cells.

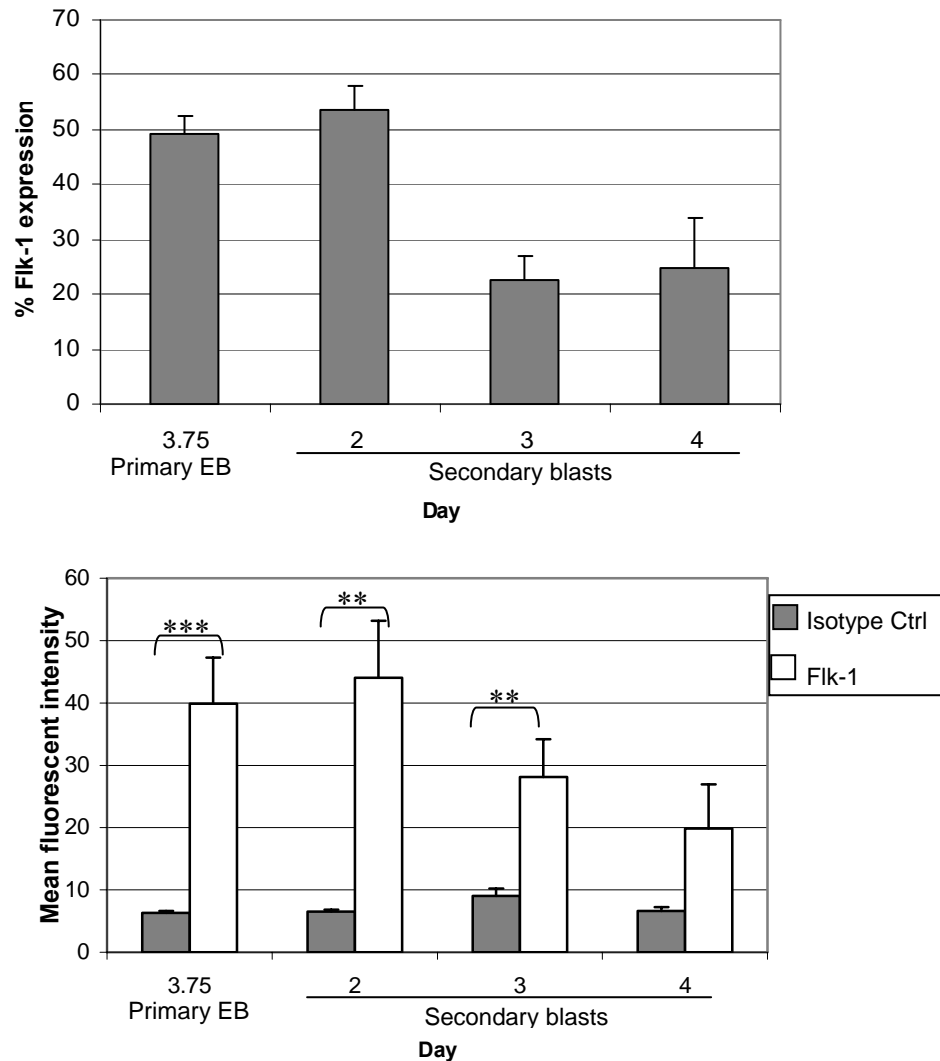


Figure 3-5. Flk-1 expression in dissociated secondary blast cells. Secondary blasts were derived from re-plated dissociated day 3.75 E14tg2A primary embryoid bodies and expanded for between 2 and 4 days. The blast colonies were then dissociated and constituent cells stained with either an anti-Flk-1 antibody or an equal concentration of isotype matched control, and a secondary FITC-labelled antibody prior to flow cytometric analysis on a Becton Dickinson FACS Vantage flow cytometer. The percentage of cells expressing Flk-1 and geometric mean intensity were calculated as described in section 3.2.1. The percentage of Flk-1 expression was calculated using a 5% marker gate. Results shown represent pooled data from 4 individual experiments. **A.** The percentage Flk-1 expression on day 3.75 EBs and day 2-4 secondary blast-derived cells are shown. **B.** Comparison of the geometric mean fluorescent intensities between the isotype control and anti-flk-1 labelled cells in day 3.75 EBs and day 2-4 secondary blast-derived cells are shown. Error bars represent s.e.m, ** = $p \leq 0.05$ in the paired students t-test. Analysis of variance (ANOVA) followed by Tukey's post-hoc test on geometric mean fluorescent intensity data showed a significant decrease in expression by day 3 (≤ 0.01). No significant decrease was demonstrated between days 3 and 4.

The time course of Flk-1 expression by secondary blast-derived cells illustrated in Figure 3-5, demonstrated a significant decrease in Flk-1 expression between days 2 and 3 of blast colony formation coinciding with the appearance of haemopoietic clusters on the periphery of the blast colony. This indicates that the down-regulation of Flk-1 may be due to haemopoietic commitment. These results correlate well with data from D'Souza *et. al.*, who observed a rapid decrease in Flk-1 cDNA in blast colonies between days 1 and 2 using Northern Blotting (D'Souza et al., 2005). The difference in timing of this decrease may be due in part to the use of different ES cell lines but is probably due in the main to the greater stability and longevity of the protein.

3.2.4 Effect of VEGF on Flk-1 expression in primary embryoid bodies

Recent work in human endothelial cells has demonstrated that unlike other receptor tyrosine kinases VEGF treatment does not increase the rate of Flk-1/KDR receptor internalisation. In fact, in short term stimulation assays VEGF stimulation actually increased KDR (kinase domain receptor, human Flk-1) recycling to the membrane from intracellular vesicles thus increasing extracellular expression (Gampel et al., 2006). To investigate whether longer term VEGF signalling in early development may affect surface expression of Flk-1, either through increasing trafficking of the receptor to the membrane or by directing differentiation towards a Flk-1 expressing population, E14tg2A ES cells were allowed to differentiate into primary embryoid bodies in methylcellulose in the presence or absence of 50ng/ml VEGF over 5 days. Flk-1 surface expression on the cells of dissociated embryoid bodies was then analysed using flow cytometry.

The data in Figure 3-6 shows a significant increase in Flk-1 surface expression cells from dissociated day 5 primary embryoid bodies cultured for 5 days in the presence of 50ng/ml exogenous VEGF. This correlates well with findings by Gampel *et al.*, (Gampel et al., 2006) in shorter term experiments. However, it is unclear whether the increase in Flk-1 expression represents increased receptor expression by individual cells or an increase in differentiation of cells within the embryoid body towards the BL-CFC/endothelial lineage.

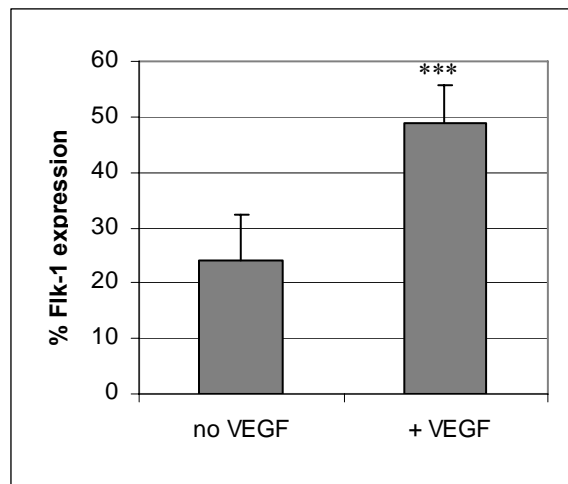


Figure 3-6 Effect of VEGF on surface expression of Flk-1. E14tg2A ES cells were allowed to differentiate into primary embryoid bodies over 5 days in the presence and absence of 50ng/ml VEGF. After 5 days dissociated embryoid body cells were stained with an anti-Flk-1 antibody or an equal concentration of isotype-matched control, and a secondary FITC-labelled antibody prior to flow cytometric analysis on a Beckton Dickinson FACS Vantage flow cytometer. The percentage of Flk-1 expression was calculated using a 5% marker as described in section 3.2.1. Results show pooled data from 3 individual experiments. Error bars represent s.e.m, *** $p \leq 0.01$ in the unpaired students t-test.

3.3 VEGF-induced Flk-1 signalling

Flk-1 plays an important role in the development and differentiation of the common precursor of both haemopoietic and endothelial development and knowledge of Flk-1-mediated signalling could provide further insight into mechanisms by which these cell types are specified. Although signalling mediated by the human homologue of Flk-1, KDR in human endothelial cells is relatively well characterised there is no evidence to suggest activation of the same pathways in Flk-1-expressing dissociated primary embryoid body cells.

VEGF stimulation of dissociated embryoid body and secondary blast cells expressing Flk-1 was used to model and investigate signalling at specific stages of development. Cells were stimulated with VEGF and proteins in the resulting whole cell lysates were separated using SDS-PAGE before being blotted onto nitrocellulose paper. The membranes were probed with antibodies that specifically detect phosphorylated signalling molecules. The duration of serum starvation of cells prior to stimulation and the concentration of VEGF used for stimulations were optimised to allow the

maximal window for signal visualisation. Cells were serum starved for 3 hours to reduce basal signalling and subsequently stimulated with 20ng/ml mVEGF.

3.3.1 VEGF induced protein phosphorylation

Flk-1 is a receptor tyrosine kinase. Upon activation with VEGF, Flk-1 transphosphorylates tyrosine residues within its cytoplasmic tail, initiating activation of phosphorylation-dependent signalling cascades. For initial characterisation of VEGF-induced Flk-1-mediated phosphorylation events day 5 embryoid body cells expressing Flk-1 were stimulated with VEGF over a 30 minute time-course. VEGF stimulated whole cell lysates were blotted and probed for proteins containing phosphorylated tyrosine residues using the 4G10 anti-phospho-tyrosine antibody.

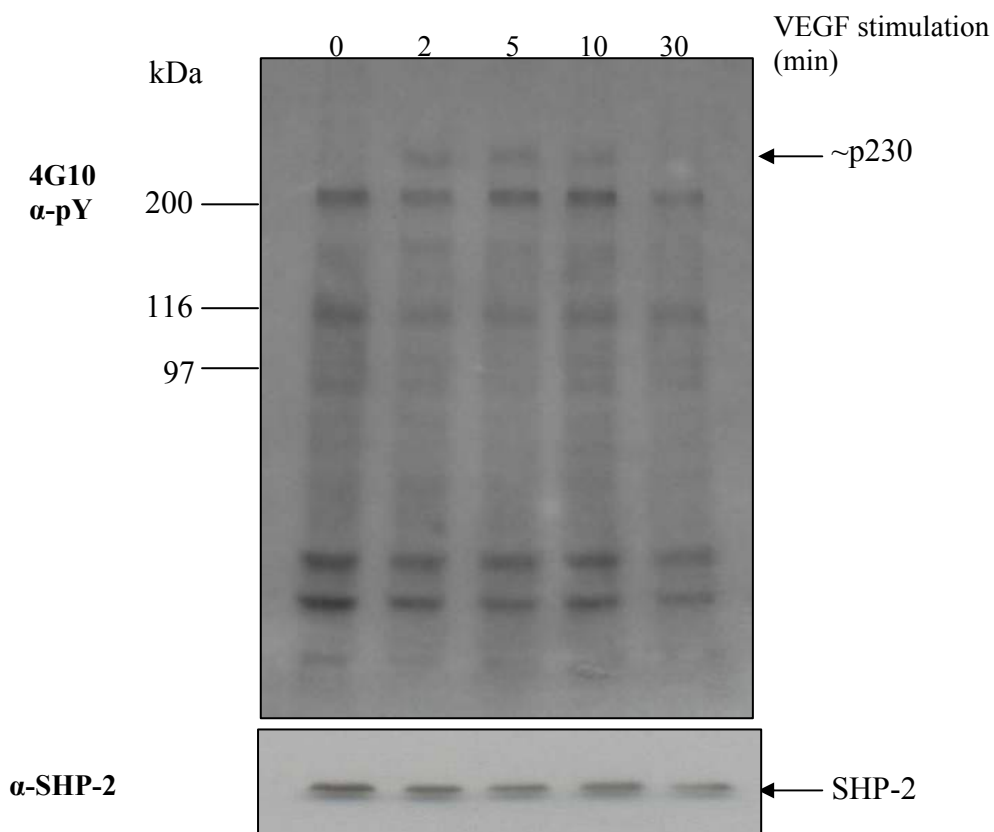


Figure 3-7 VEGF-stimulated Flk-1-mediated tyrosine phosphorylation. Immunoblot of whole cell lysates from day 5-dissociated primary EB cells stimulated with 20ng/ml VEGF over a 30 minute time-course and probed with the anti-phosphotyrosine antibody 4G10. Representative of two individual experiments. The blot was re-probed with the anti-SHP-2 antibody to control for equal protein loading. Molecular markers are illustrated at 97,116 and 200kDa

Figure 3-7 demonstrates few proteins for which tyrosine phosphorylation increases upon VEGF stimulation. The only protein for which tyrosine phosphorylation appears to increase is the protein at ~ 230kDa. This is the approximate size of the Flk-1 receptor itself so this increase in phosphorylation probably represents transphosphorylation of tyrosines on the cytoplasmic tail of the receptor.

3.3.2 Effect of VEGF on MAPK signalling

In adult endothelial cells VEGF stimulation has been shown to activate the Mitogen Activated Protein Kinase (MAPK) pathway, which is important for growth, differentiation and development in endothelial cells (Doanes et al., 1999; Glikli et al., 2001; Takahashi and Shibuya, 1997) (see section 1.4.2.2). Activation of this pathway can be monitored by the phosphorylation state of the downstream Extracellular signal Regulated Kinases 1/2 protein (ERK1 and 2), which require phosphorylation on threonine 202 and tyrosine 204 for full activation. ERK1/2 are usually activated only transiently and turn on the ‘immediate early genes’ within minutes (Lodish et al., 1999). Previous work in rat sinusoidal endothelial cells has demonstrated that VEGF-induced ERK activation peaked between 3 and 5 minutes and declined to near basal levels after 10 minutes (Takahashi and Shibuya, 1997). Little work has taken place to characterise the existence (or kinetics) of Flk-1-induced MAPK activation in early developmental models. A single study performed a 30 minute VEGF stimulation of day 3 embryoid body cells expressing Flk-1 and demonstrated VEGF-induced phosphorylation of ERK1/2 (Park et al., 2004). However, as noted above, maximal ERK phosphorylation upon cytokine stimulation generally occurs over a shorter time course. To confirm Flk-1-mediated MAPK activation and to establish its kinetics a time-course of VEGF stimulation was therefore performed in embryoid body and secondary blast-derived cells.

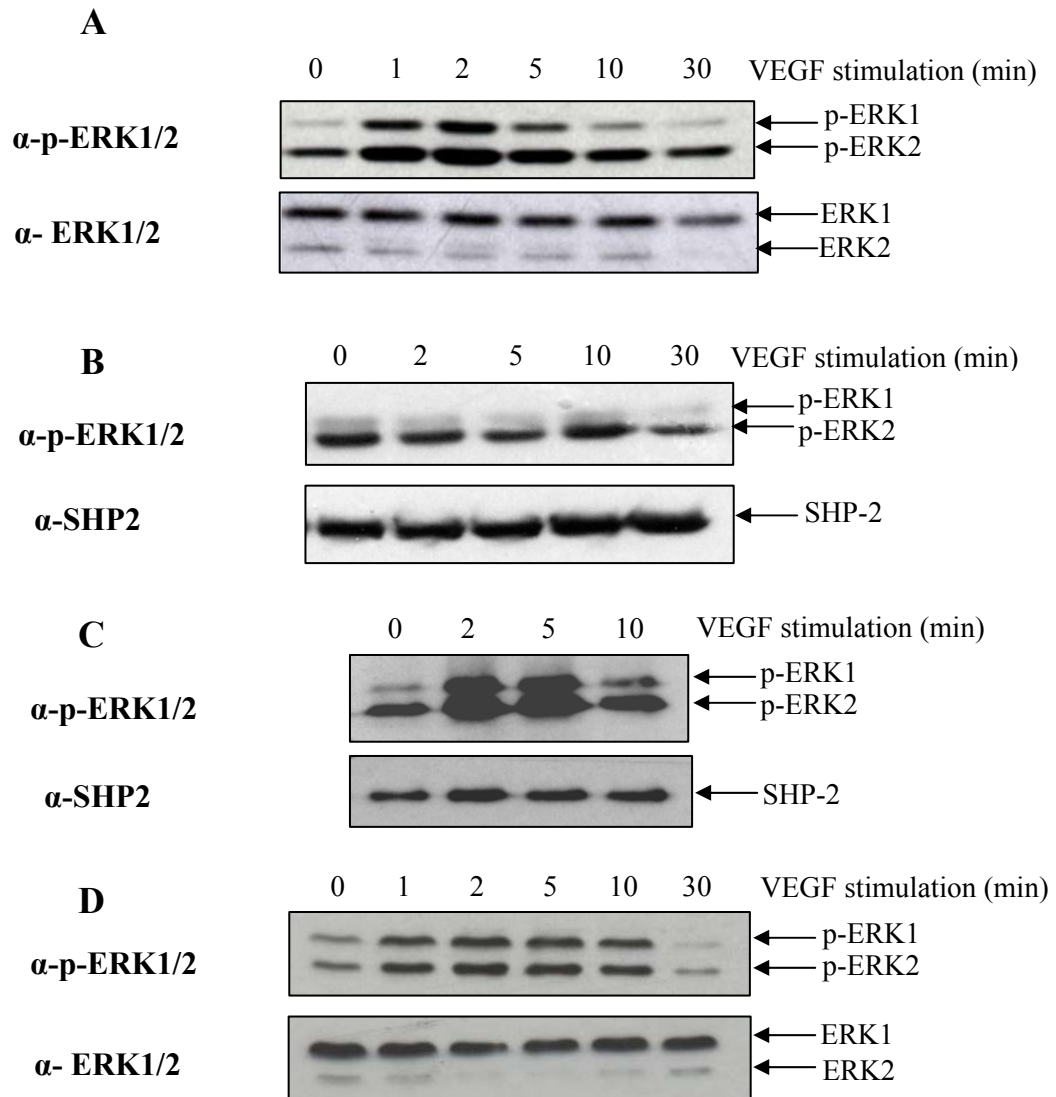


Figure 3-8 VEGF- induced MAPK signalling. Primary embryoid bodies and secondary blasts were dissociated with trypsin-EDTA, D4T cells were stimulated on the Petri dishes in which they were cultured. Cells were serum-starved for 3 hours at 37°C prior to stimulation with 20ng/ml VEGF for between 1 and 30 minutes as indicated. Immunoblotting was performed and blots were probed with an anti-p-ERK1/2 antibody then stripped and reprobed with either anti-SHP-2 or anti-ERK1/2 antibodies to check for equal loading. **A.** Immunoblot of day 5 dissociated E14tg2A primary embryoid bodies stimulated with VEGF. The blot shown is representative of 5 individual experiments. **B.** Immunoblot of day 5 dissociated R1Flk-1^{-/-} primary embryoid bodies stimulated with VEGF. The blot shown is representative of 2 individual experiments. **C.** Immunoblot of day 2 dissociated secondary blast cells stimulated with VEGF. The blot shown is representative of 3 individual experiments. **D.** Immunoblot of D4T embryoid body derived endothelial cells stimulated with VEGF. The blot shown is representative of 3 individual experiments.

Initial experiments demonstrated that ERK1/2 was phosphorylated by VEGF stimulation in primary embryoid bodies, secondary blasts and D4T embryoid body-derived endothelial cells (included as control) (Figure 3-8A, C and D). The observed increases in ERK phosphorylation were transient, occurring within 1 minute, peaking between 2 and 5 minutes and decreasing back to basal levels by between 5 and 10 minutes of VEGF stimulation. Day 4 R1 embryoid bodies yielded similar results (data not shown). The VEGF-induced ERK phosphorylation profile is typical of ERK1/2 activation and correlates well with previous work in rat sinusoidal endothelial cells (Takahashi and Shibuya, 1997). However, an increase in ERK1/2 phosphorylation over basal was not visualised upon 30 minutes VEGF stimulation, in contrast to findings by Park *et al.* (Park et al., 2004).

To verify that VEGF was acting to induce ERK1/2 phosphorylation through the Flk-1 receptor the experiment was repeated using day 4 R1Flk-1^{-/-} embryoid bodies (Figure 3-8B). VEGF stimulation provoked no increase in ERK phosphorylation confirming that VEGF was acting through the Flk-1 receptor.

3.3.3 Is VEGF-mediated ERK signalling activated via PLC γ ?

Work in HUVECs suggests that in adult human endothelial cells VEGF-induced MAPK activation occurs through a phospholipase C- γ (PLC γ)-dependent Protein Kinase C (PKC)-mediated mechanism rather than the classical Ras-Raf pathway (Doanes et al., 1999; Glikli et al., 2001; Yashima et al., 2001). However, further work by Yashima *et al.*, demonstrated that the mechanism of VEGF activation of the MAPK pathway may be heterogenous between endothelial cells of different origins. Whilst VEGF appeared to activate the MAPK pathway through a PKC-mediated mechanism in HUVECs and human aortic endothelial cells (HAECs), in human subcutaneous microvascular endothelial cells (HMVECs) MAPK activation seemed dependent only on the classical Ras-Raf pathway (Yashima et al., 2001).

In order to elucidate any potential role for PLC γ in Flk-1-mediated MAPK activation, VEGF activation of PKC (a downstream effector of PLC γ) in primary

embryoid body-derived cells was first investigated. There are three classes of PKC isoforms; classical, novel and atypical all of which require phosphorylation for their activation (Newton, 2003). In addition to phosphorylation, activation of the classical isoforms (α , β I, β II and γ) is dependent upon both calcium and Diacylglycerol (DAG). The novel isoforms (δ , η , θ and ϵ) do not require calcium for their activation but are still dependent upon DAG and the atypical isoforms (λ and ζ) require only phosphorylation for their activation (Violin and Newton, 2003). VEGF-stimulated primary embryoid body-derived cell lysates were immunoblotted and blots were probed with antibodies specific for phosphorylated classical and novel PKC isoforms and phosphorylated PKC- δ (implicated in VEGF-induced MAPK activation in HUVECs (Gliki et al., 2001)).

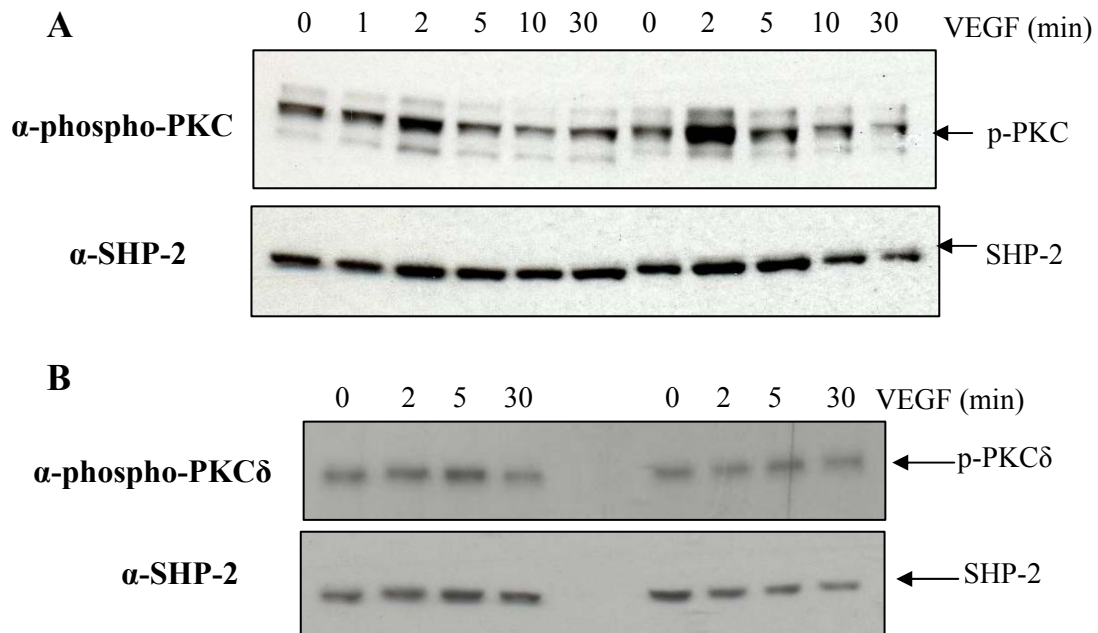


Figure 3-9 Effect of VEGF stimulation on phosphorylation of classical and novel PKC isoforms and more specifically phosphorylation of the novel isoform PKC δ . Dissociated day 5 embryoid body cells were serum-starved for 3 hours then stimulated with 20ng/ml VEGF for between 1 and 30 minutes as indicated. Immunoblotting was performed and blots were probed with either an anti-phospho-PKC which detects phosphorylation of the classical and novel PKC isoforms or an anti-phospho-PKC δ antibody then stripped and reprobed with an anti-SHP-2 antibody to check for equal loading of protein. 2 individual experiments are illustrated in both **A** and **B**.

Figure 3-9A illustrates an increase in total phosphorylation of classical and novel PKC isoforms after 2 minutes of VEGF stimulation which decreases back to basal levels by 5 minutes. No obvious increase in PKC δ phosphorylation over basal levels was visualised upon VEGF stimulation (Figure 3-9B).

To investigate whether the VEGF-induced PKC activation observed in Figure 3-9A mediates downstream MAPK activation, Calphostin C, a pan-PKC inhibitor derived from *Cladosporium Cladosporioides* and gö6976, an inhibitor of the classical calcium dependent class of PKCs consisting PKC α , β_1 and β_2 which also inhibits PKC μ (PKD) were used. Primary embryoid bodies were dissociated and serum starved for 3 hours. They were then incubated with Calphostin C or gö6976 for a further 45 minutes prior to stimulation with 20ng/ml VEGF. MAPK activation was examined using immunoblots of the cell lysates. ERK1/2 phosphorylation in Figure 3-10 does not appear to decrease in the presence of Calphostin C applied at 250nM or 1 μ M (the concentration used by Glikli *et al.*, to demonstrate PKC reliance of KDR induced MAPK activation in HUVECs). However, a slight decrease in ERK1/2 phosphorylation is seen in lysates from cells preincubated with 3 μ M gö6979 prior to VEGF stimulation. At this concentration *in vitro* studies using isolated proteins showed only inhibition of PKC α β I, β II γ or PKD (Martiny-Baron *et al.*, 1993) but selectivity over other kinases is unclear. If activation of the MAPK pathway by Flk-1 were mediated through any PKC isoform a decrease in ERK phosphorylation would be expected upon inhibition of the classical and atypical PKC isoforms with Calphostin C. As no such decrease was observed it appears likely that in embryoid body-derived cells activation of the MAPK pathway by Flk-1 is mediated through the classical Ras-Raf pathway.

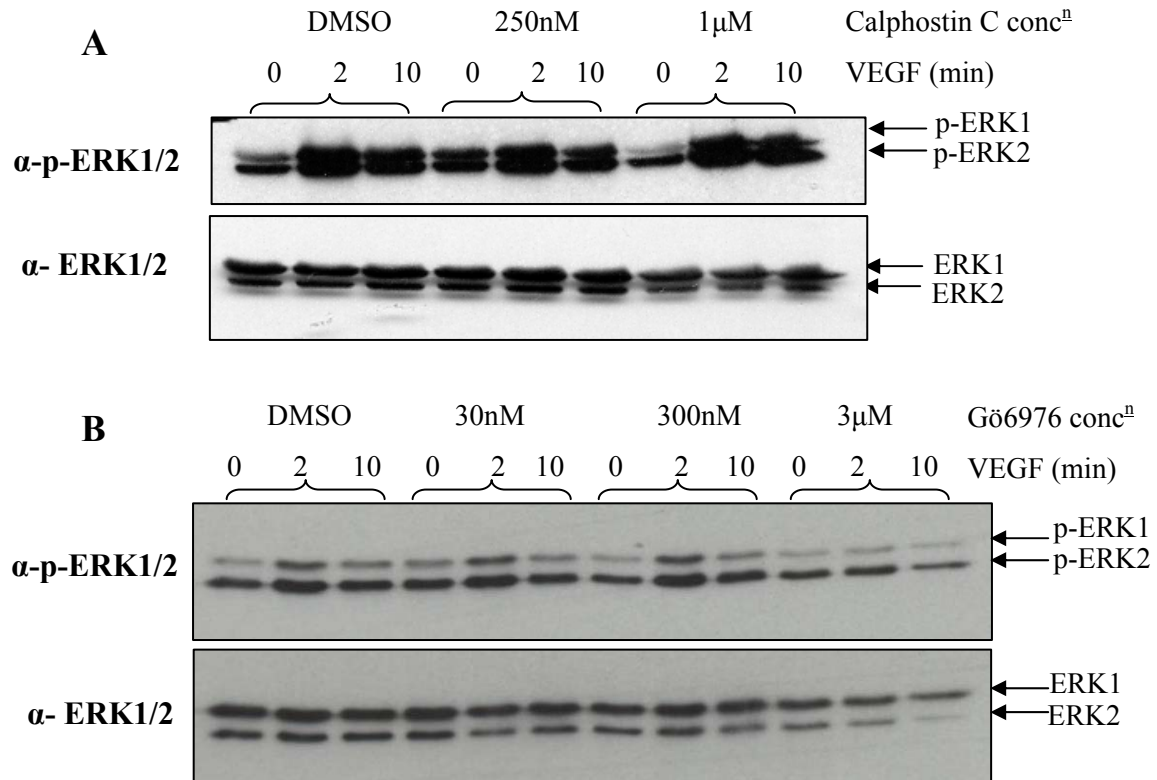


Figure 3-10 Effect of PKC inhibition on ERK1/2 phosphorylation. Dissociated day 5 embryoid bodies were serum-starved for 3 hours prior to a 45 minute incubation with a DMSO control, Calphostin C (**A**) or gö6979 (**B**). Cells were then stimulated with 20ng/ml VEGF for 0, 2 or 10 minutes. Immunoblotting was performed and blots were probed with an anti-phospho ERK1/2 antibody then reprobbed with an anti-ERK1/2 antibody to check for equal loading. **A** is representative of 2 individual experiments. **B** is an individual experiment.

3.3.4 Effect of VEGF stimulation on PI3K signalling

In adult endothelial cells VEGF stimulation has been shown to activate the class IA Phosphoinositide-3-kinase (PI3K) pathway, thought to be involved in cell proliferation (Dayanir et al., 2001; Hamada et al., 2005), survival (Fujio and Walsh, 1999; Gerber et al., 1998), migration (Holmqvist et al., 2004) and eNOS activation (Bates and Harper, 2002; Fulton et al., 1999; Lin and Sessa, 2006). Class IA PI3K isoforms (see section 1.4.2.3) are expressed from the single cell stage of development and play an important role in early developmental processes (Lu et al., 2004; Riley et al., 2005) including haemopoiesis (Bone and Welham, 2007; Clayton et al., 2002; Haneline et al., 2003; Okkenhaug et al., 2002). An endothelial cell specific *Tie2CrePTEN* construct has been used to investigate the role of PI3K signalling in

endothelial cells during development. Animals deficient in PTEN, a phosphatase which negatively regulates PI3K signalling, died at E11.5 due to bleeding and cardiac failure caused by impaired recruitment of pericytes to the blood vessels and cardiomyocytes to the heart. Studies *in vitro*, and using the heterozygous mutant, showed enhanced proliferation of endothelial cells in the PTEN mutant suggesting a proliferative role for PI3K in the endothelium (Hamada et al., 2005).

Activation of PI3K signalling can be monitored by the phosphorylation status of the downstream mediators of the PI3K pathway including protein kinase B (PKB), Glycogen Synthase Kinase-3- α/β (GSK-3- α/β), and S6 ribosomal protein (see Figure 1-17). The PI3K pathway is usually activated transiently and can directly regulate apoptosis, migration, the cell cycle and translation (Lodish et al., 1999). Previous work in bovine aortic endothelial cells (BAECs), porcine aortic endothelial cells (PAEs) and HUVECs has demonstrated maximal VEGF-induced PKB/Akt phosphorylation between 7.5 and 30 minutes of treatment declining thereafter (Bernatchez et al., 2001; Dayanir et al., 2001; Gerber et al., 1998). Little work has taken place to characterise the existence (or kinetics) of Flk-1-mediated PI3K activation in early developmental models. A single study performed a 30 minute VEGF stimulation of day 3 embryoid body cells expressing Flk-1 but did not observe any VEGF-induced increase in PKB/Akt phosphorylation (Park et al., 2004). However, as noted above, maximal PI3K phosphorylation upon cytokine stimulation may occur over a shorter time-course. To investigate if VEGF/ Flk-1 mediate PI3K activation in an *in vitro* model of early development a time-course of VEGF stimulation was performed using both embryoid body and secondary blast-derived cells. The D4T embryoid body-derived endothelial cell line and HUVECs were also stimulated as controls. Activation of the PI3K pathway was probed using the following antibodies which recognise phosphorylated downstream effectors of PI3K:

α -phospho-PKB – detects endogenous levels of Akt1, only when phosphorylated at Ser473.

α -phospho-GSK-3 α/β – detects endogenous levels of GSK-3, only when phosphorylated at Ser21 of GSK-3 α or Ser9 of GSK-3 β .

α -phospho-GSK-3 β – detects endogenous levels of Ser9 of GSK-3 β .

α -phospho-PKB substrate – Detects peptides and proteins containing phosphor-Ser/Thr preceded by Lys/Arg at positions -5 and -3.

α -phospho-S6 – detects endogenous levels of S6 ribosomal proteins, only when phosphorylated at Ser235 and Ser236.

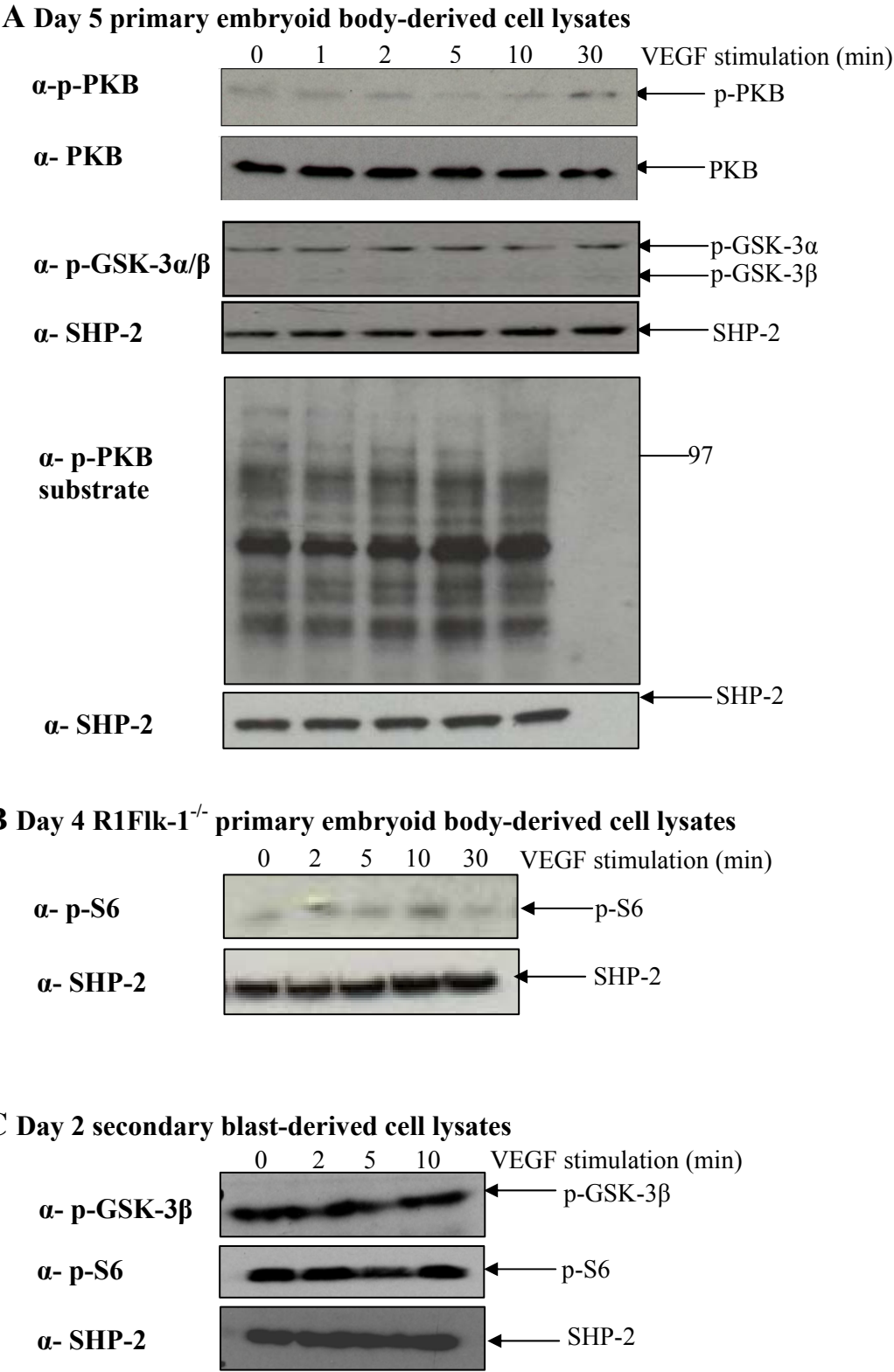


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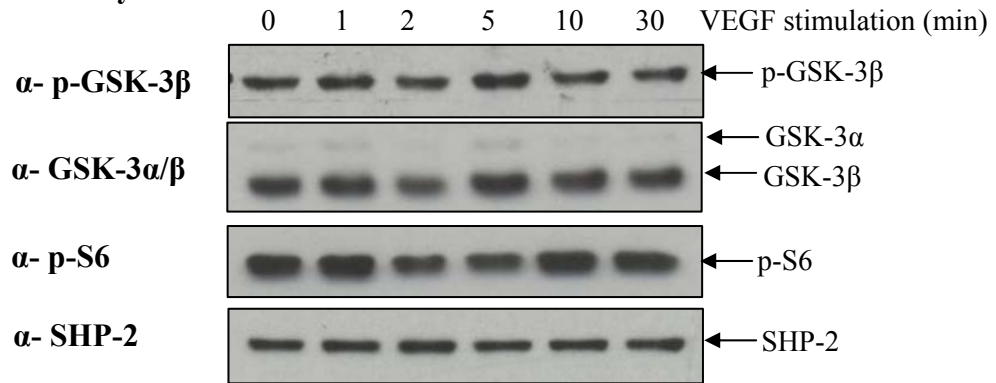
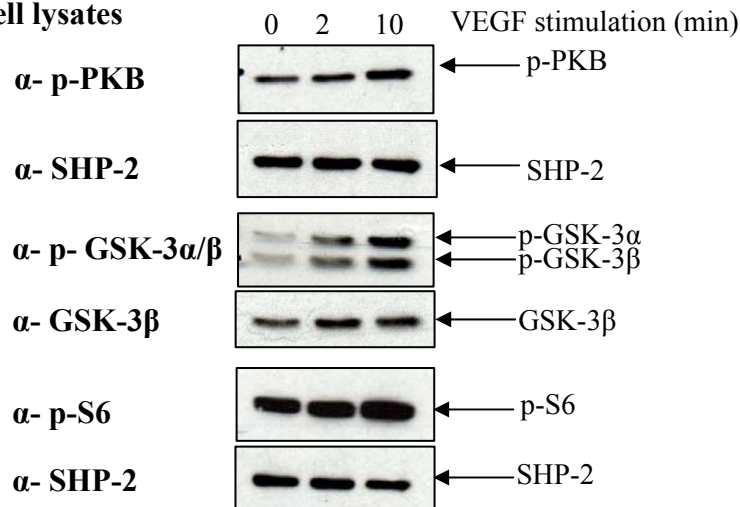
D D4T cell lysates**E HUVEC cell lysates**

Figure 3-11 Effect of VEGF stimulation on phosphorylation status of downstream mediators of PI3K. Primary embryoid bodies and secondary blasts were dissociated with trypsin-EDTA, D4T cells and HUVECs were stimulated in the Petri dishes in which they were cultured. Cells were serum-starved for 3 hours at 37°C prior to stimulation with 20ng/ml VEGF for between 1 and 30 minutes as indicated. Immunoblotting was performed and blots were probed with anti-p-PKB, anti-p-GSK-3 α and β , anti-p-PKB substrate and anti-p-S6 antibodies then stripped and re-probed with anti-PKB, anti-GSK-3 α/β or SHP-2 antibodies to check for equal loading. **A.** Immunoblot of day 5 dissociated E14tg2A primary embryoid bodies stimulated with VEGF. The blots shown are representative of individual experiments as follows α -p-PKB n=3, α -p-GSK-3 n=5, α -p-PKB substrate n=2. **B.** Immunoblot of dissociated day 4 R1Flk-1^{-/-} embryoid bodies stimulated with VEGF. **C.** Immunoblot of day 2 dissociated secondary blast cells stimulated with VEGF. The blots shown are representative of 2 individual experiments. **D.** Immunoblot of D4T embryoid body derived endothelial cells stimulated with VEGF. The blots shown are representative of 3 individual experiments. **E.** Immunoblot of HUVECs stimulated with VEGF. The blots shown are representative of 2 individual experiments.

The levels of phosphorylation of neither PKB, GSK-3 α/β nor S6 ribosomal protein showed any consistent change upon VEGF stimulation over a 30 minute time-course in primary embryoid bodies (Figure 3-11A), secondary blasts (Figure 3-11C) or D4T embryoid body derived endothelial cells (Figure 3-11D). These results correlate with the lack of PKB phosphorylation seen upon 30 minute VEGF stimulation in day 3 primary embryoid bodies (Park et al., 2004). However, Figure 3-11E shows that VEGF stimulation of HUVECs *did* elicit increased phosphorylation of all three downstream mediators as previously described (Gerber et al., 1998) demonstrating that this was not due to the methodology used. Basal phosphorylation levels of GSK-3 α/β and S6 were high demonstrating that the PI3K is active in these cells. This high basal level was also observed α p-S6 blots in VEGF-stimulated day 4 Flk-1^{-/-}R1 EB-derived cells suggesting it is not due to endogenous activation of PI3K through the Flk-1 receptor (Figure 3-11B).

3.3.5 Effect of PI3K and MEK inhibition on VEGF/Flk-1-mediated MAPK and PI3K signalling

Many papers have been published discussing the existence of cross-talk between the PI3K and MAPK pathways in different cell systems. Work in ES cell self-renewal has shown that PI3K inhibition increases ERK phosphorylation implying that PI3K negatively regulates the MAPK pathway in ES cells (Paling et al., 2004). In the vascular endothelium PI3K has been shown to play an inhibitory role in VEGF-induced MAPK-dependent tissue factor expression (Blum et al., 2001). More recently, a study in zebrafish has implicated opposing roles of VEGF-induced PI3K and MAPK activation as important for endothelial specification towards either an arterial (MAPK) or venous (PI3K) fate (Hong et al., 2006). It is thought that this cross-talk may be mediated by PKB phosphorylation and inactivation of Raf, an upstream effector of MAPK signalling in many cell types (Zimmermann and Moelling, 1999). However, in other cell types there is no such evidence of cross-talk (Chiu et al., 2005) and in some cases PI3K appears to activate rather than inhibit MAPK signalling (Bhatt et al., 2002). To investigate any potential cross-talk between these two pathways upon VEGF stimulation EB-derived cells were incubated with specific inhibitors of each pathway prior to VEGF stimulation and the effects on

phosphorylation of downstream effectors were monitored. U0126, a non-competitive specific inhibitor of MEK1 and MEK2 activity (Favata et al., 1998), was used to inhibit the MAPK pathway and LY294002 (Vlahos et al., 1994), a reversible competitive inhibitor of PI3K with a 19-fold selectivity for class IA over class II PI3K, was used to inhibit the class IA PI3K pathway.

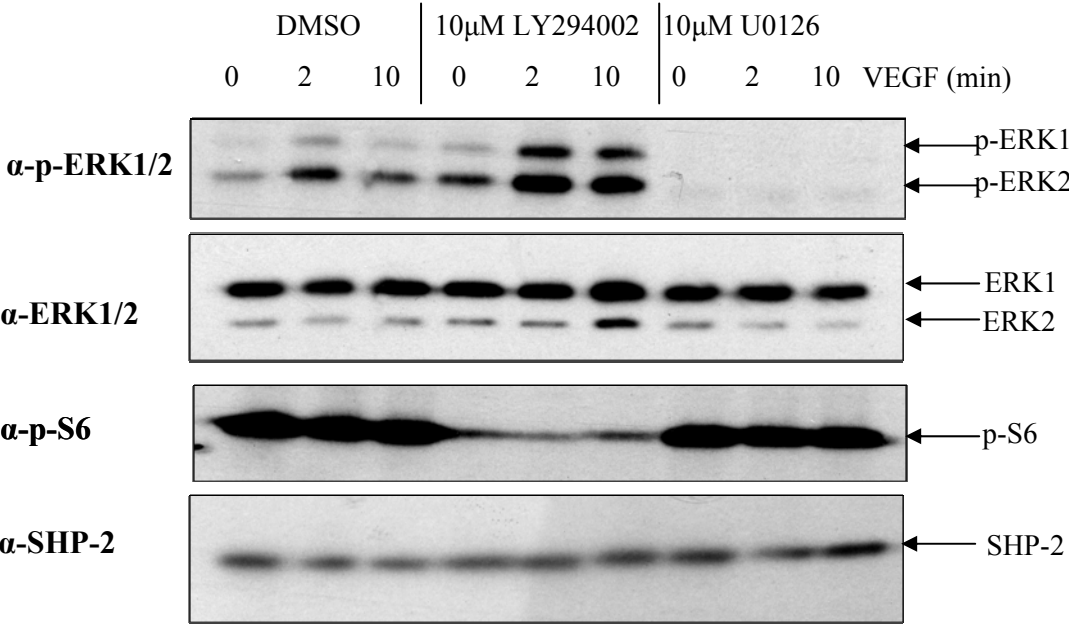


Figure 3-12 Effect of PI3K and MAPK inhibition on VEGF-stimulated ERK1/2 activation. Dissociated day 5 embryoid bodies were serum-starved for 3 hours prior to a 45 minute incubation with a DMSO control, 10μM U0126 (MAPK inhibitor) or 10μM LY294002 (PI3K inhibitor). Cells were then stimulated with 20ng/ml VEGF for 0, 2 or 10 minutes. Immunoblotting was performed and blots were probed with either anti-phospho ERK1/2 or anti-phospho-S6 ribosomal protein antibodies then stripped and reprobed with anti-ERK1/2 and anti-SHP-2 antibodies to check for equal loading. The blots shown are representative of 3 individual experiments

Figure 3-12 illustrates that as expected, incubation of the EB-derived cells with the MEK1/2 inhibitor U0126 prior to VEGF stimulation abrogated VEGF-induced ERK1/2 phosphorylation. However, pre-treatment with U0126 appeared to have no effect on S6 phosphorylation. Incubation of the cells with the PI3K inhibitor LY294002 prior to VEGF stimulation abrogated S6 phosphorylation but also appeared to increase ERK phosphorylation suggesting that the PI3K pathway is acting to negatively regulate the MAPK pathway in these cells (as we reported in Paling *et al.*, in 2004 in ES cells). These findings correlates well with data from both

adult endothelial cells and angioblasts undergoing commitment to either arterial or venous fates in which VEGF-induced MAPK activation increases upon inhibition of the PI3K pathway with either LY294002 or an irreversible highly selective PI3K inhibitor, wortmannin (Blum et al., 2001; Hong et al., 2006).

3.4 Discussion

Primary embryoid bodies and secondary blasts were used as models of early development. Their constituent cells were used to study Flk-1 expression and VEGF-induced signalling. Results are summarised below and in Figure 3-13:

3.4.1 Summary

- Flk-1 is expressed in dissociated primary E14tg2A embryoid body cells from day 3.5 of formation with expression peaking between days 4 and 6. In R1 dissociated embryoid body cells Flk-1 is expressed from day 3 peaking between days 4 and 5.
- Flk-1 expression in secondary blast-derived cells declines upon blast expansion. In contrast to expression in primary embryoid body-derived cells expression in secondary blast-derived cells occurs within a restricted population.
- VEGF stimulation of primary embryoid body-derived cells and secondary blast-derived cells results in auto-phosphorylation of Flk-1 and activation of the MAPK pathway (demonstrated by ERK1/2 phosphorylation).
- VEGF stimulation of Primary embryoid body-derived cells also activates the PLC γ 1 pathway (demonstrated by PKC phosphorylation).
- Preliminary evidence suggests that activation of the MAPK pathway in primary embryoid body-derived cells is not mediated by PLC γ 1.

- VEGF stimulation of Flk-1 expressing primary embryoid body-derived cells or secondary blast-derived cells does not appear to activate signalling through PI3K.
- Inhibition of the PI3K pathway with the pharmacological inhibitor LY294002 reduces phosphorylation of the downstream mediator S6 ribosomal protein and increases VEGF-induced ERK1/2 phosphorylation.
- Inhibition of the MAPK pathway with the pharmacological inhibitor of MEK1/2, U0126, abolishes phosphorylation of their downstream target ERK1/2 but has no effect on PI3K signalling, measured by the phosphorylation status of S6 ribosomal protein.

3.4.2 Flk-1 expression

3.4.2.1 *Flk-1 expression in dissociated Primary embryoid body cells*

Flk-1 surface expression on primary embryoid body-derived cells, measured by flow cytometry, was very heterogenous with a range of expression levels within the population. Flk-1 expression profiles differed between the two ES cell lines used. Differences in Flk-1 expression profiles between groups using different ES cell lines have been previously reported and it appears that these may in part be due to differences in the lines themselves in addition to differences in the differentiation protocols used (Chung et al., 2002; Fehling et al., 2003; Kabrun et al., 1997). In E14tg2A primary embryoid bodies Flk-1 expression first arose between day 3 and 3.5, peaked between days 4 and 6 and declined thereafter. In R1 primary embryoid bodies Flk-1 expression was seen earlier at day 3 after plating, expression peaked at day 4 and declined thereafter, closely correlating with previous Flk-1 expression profiles performed using these cells (Chung et al., 2002). Though there was a slight temporal difference in Flk-1 expression, which points to slower differentiation in the E14tg2A ES cell line vs the R1 ES cell line, the overall pattern of Flk-1 expression in both cell lines followed previously published *in vitro* data (Chung et al., 2002; Kabrun et al., 1997). Leahy *et al.*, ascribe day 3.5-4.5 of embryoid body development as equivalent to day 6.5-7.5 of embryonic development. Flk-1 is first expressed at

around day 7 in the embryo, (Leahy et al., 1999) therefore the up-regulation of Flk-1 in both E14tg2A and R1 EB-derived cells also correlates well with its reported expression in the embryo proper.

Early Flk-1 expression marks the haemangioblast, the common precursor of both haemopoietic and endothelial cells, and its *in vitro* equivalent the Blast Colony Forming Cell (BL-CFC) (Fehling et al., 2003; Huber et al., 2004). This suggests that early expression (day 3-4.5 in R1 EBs and day 3.5-5 in E14tg2A EBs) marks this transient population. Thereafter it is likely that the declining Flk-1 population largely represents cells of the endothelial lineage as by day 6 of embryoid body development Flk-1 is reportedly lost from the haemopoietic lineages (Kabrun et al., 1997).

3.4.2.2 Flk-1 expression in dissociated secondary blast cells

Flk-1 expression in secondary blast-derived cells was more homogenous than that of primary embryoid body-derived cells with clear positive and negative populations. Initially, expression also appeared to occur at higher levels on a greater proportion of cells, probably due to the culture conditions allowing preferential expansion of the BL-CFC over other cell populations which had developed within the embryoid bodies. Flk-1 expression in these cells remained elevated until day 2 of blast colony formation and declined thereafter. Blast colony development has been described as a sequential process involving both proliferation and differentiation events. During the first 2 days of blast colony formation the BL-CFC proliferates forming a tight ball of cells (D'Souza et al., 2005). During this time surface expression of Flk-1, itself a marker of the BL-CFC, remains elevated. Haemopoietic cells then begin to develop around this core. The mature blast colony consists of a vascular smooth muscle and endothelial core with haemopoietic cells clustered around the periphery (D'Souza et al., 2005). The decline in Flk-1 expression from day 3 of blast colony formation correlates with the timing of blast expansion and confirms the decrease in Flk-1 determined using gene expression analysis by D'Souza *et al.*, who observed a rapid decrease in Flk-1 cDNA in blast colonies between days 1 and 2 (D'Souza et al., 2005). The difference in timing of this decrease may be due in part to the use of different ES cell lines but is probably due in the main to the greater stability and longevity of the protein, compared to RNA. The down-regulation of Flk-1 expression

upon blast expansion is probably due to its reported down-regulation upon haemopoietic commitment (Kabrun et al., 1997). The surface expression that remains presumably lies in the non-committed secondary blast population and in committed endothelial cells.

3.4.2.3 Effect of culture in VEGF on Flk-1 expression in dissociated day 5 primary embryoid body-derived cells

Primary embryoid bodies were cultured for 5 days in the presence or absence of 50ng/ml exogenous VEGF to investigate any possible effects of VEGF on Flk-1 expression. Day 5 primary embryoid body-derived cells showed a modest but significant increase in the percentage of cells expressing Flk-1 on their surface in the presence of exogenous VEGF. Possible explanations for this are; that the addition of VEGF to the developing embryoid bodies directed differentiation towards the BL-CFC and endothelial lineages meaning that a greater proportion of cells in the embryoid body expressed Flk-1, or that addition of VEGF increased the amount of Flk-1 receptor expressed by cells within the Flk-1 positive population. However, because Flk-1 expression is so heterogenous within primary embryoid body-derived cells and the increase in Flk-1 surface expression is numerically small it is difficult to ascertain which of these hypotheses may be correct.

Interestingly, the observed increase in Flk-1 expression upon culture in VEGF correlates well with the increase of surface Flk-1 expression in human adult endothelial cells upon shorter term VEGF stimulation reported recently (Gampel et al., 2006). Gampel *et al.*, reported that unlike in other receptor tyrosine kinase systems, where ligand stimulation causes receptor internalisation, VEGF stimulation of Flk-1 in HUVECs and HMVECs increased the rate of constitutive receptor cycling and thus increased surface Flk-1 expression. Although the results in this study correlate well with the results from Gampel *et al.*, the increase in Flk-1 expression observed in their system was the result of shorter term assays (up to 24 hours) and it is unclear whether an increase in receptor cycling could be maintained over a longer time-course. Shorter-term VEGF stimulation studies utilising confocal microscopy would clarify whether VEGF does induce an increase in Flk-1 receptor cycling in primary embryoid body-derived cells. If this is the case it is possible that

increased receptor cycling could be mediated through activation of the MAPK pathway. Studies in neural stem cells have demonstrated that bFGF can cause up-regulation of Flk-1 surface expression through activation of the MAPK pathway (Xiao et al., 2007). In short-term stimulation experiments VEGF does stimulate Flk-1 mediated ERK1/2 activation (see Figure 3-8), it is therefore possible that this increase in Flk-1 surface expression is part of a VEGF-induced autocrine loop. The effect of ERK activation on Flk-1 expression could be evaluated using an mES cell line expressing tet-regulatable constitutively active MEK-1 (generated by Professor Melanie Welham, unpublished data) and/or by using pharmacological inhibitors of the MAPK pathway such as U0126 or PD98059 (see section 4.3.1). As the MAPK pathway is important for differentiation (Burdon et al., 1999), its constitutive activation or inhibition may well affect the timing or balance of differentiation within the embryoid body making this approach difficult. This approach may therefore be better suited to short term assays for example in the study of the effects of VEGF-stimulation on Flk-1 receptor cycling in embryoid body derived cells using confocal imaging.

An increase in Flk-1 protein expression upon addition of VEGF has been seen in rat kidney explants in longer term assays. Kidney explants from E14 rats, incubated in the presence of 5ng/ml VEGF for 6 days, demonstrated increased Flk-1 protein expression measured by immunoblotting when compared with explants cultured in the absence of VEGF (Tufro et al., 1999). Unfortunately we were unable to optimise Flk-1 immunoblotting in our system during this experiment so it is unknown whether the observed increase in Flk-1 surface expression was accompanied by an increase in total Flk-1 expression, as demonstrated by Tufro *et al.*, indicating an increase in Flk-1 transcription, or no such increase indicating that the increase in Flk-1 surface expression was due to increased receptor trafficking as observed by Gampel *et al.* Increased Flk-1 transcription could be investigated using semi-quantitative RT-PCR or quantitative PCR to monitor levels of Flk-1 gene expression in cells of embryoid bodies cultured in the presence or absence of exogenous VEGF. The effect of VEGF on Flk-1 receptor trafficking could be investigated using a similar strategy to that used by Gampel *et al.* Flk-1 positive embryoid body-derived cells could be sorted using FACS or a magnetic based cell sorting system such as the MACS system. Following short-term VEGF-stimulation cells could be fixed and permeabilised and

an anti-Flk-1 antibody could be used to study localisation of the Flk-1 receptor in VEGF stimulated and unstimulated Flk-1-expressing cells using confocal microscopy.

Initially, a more thorough characterisation of the perceived effect of VEGF on Flk-1 expression in embryoid body-derived cells might include a more complete time course of Flk-1 surface expression in the presence and absence of VEGF to assess when the increase in surface expression first occurs and whether the kinetics of Flk-1 expression are altered in the presence of exogenous VEGF. The effect of VEGF on Flk-1 surface expression may be greater if the effects of endogenous VEGF could be diminished by use of a neutralising antibody or more simply by the use of VEGF null ES cells (Ng et al., 2004)

To further dissect the mechanism by which culture of embryoid bodies with exogenous VEGF increases Flk-1 expression immunohistochemistry or confocal microscopy could be used better quantify the percentage of cells expressing Flk-1 in the presence and absence of VEGF to determine whether the overall percentage of cells expressing Flk-1 changes. If changes in the percentage of cells expressing Flk-1 were seen gene expression profiling of lineage specific genes including *Brachyury*, *Scl*, *Pecam-1*, *Pax-6*, *Nodal* and *Gata-6* could be used to further assess any effects on lineage commitment.

3.4.3 VEGF induced Flk-1 signalling

3.4.3.1 VEGF- induced protein tyrosine phosphorylation

VEGF stimulation of dissociated primary embryoid body cells did not appear to greatly affect the tyrosine phosphorylation status of proteins within the cells, at least as measured by immunoblotting with an anti-phosphotyrosine antibody. Increased tyrosine phosphorylation of a protein of ~230kDa was thought to be likely to represent autophosphorylation of the fully glycosylated Flk-1 receptor, as observed by other researchers in different cell types (Dougher and Terman, 1999; Kendall et al., 1999; Neufeld et al., 1999). This lack of obvious tyrosine phosphorylation cascades does not mean that Flk-1 is not mediating many signalling processes in these cells however, only that they are more subtle.

3.4.3.2 VEGF- induced activation of the MAPK pathway

In adult endothelial cells VEGF stimulation has been shown to activate the Mitogen Activated Protein Kinase (MAPK) pathway, which is important for growth, differentiation and development of endothelial cells (Doanes et al., 1999; Gliki et al., 2001; Takahashi and Shibuya, 1997). ERK1/2, a downstream effector of the MAPK pathway is usually activated only transiently (Lodish et al., 1999; Takahashi and Shibuya, 1997). However, only a longer term 30 minute VEGF stimulation of embryoid body derived cells had previously been reported to activate MAPK signalling (Park et al., 2004). Initial experiments demonstrated that ERK1/2 were transiently phosphorylated by VEGF stimulation of primary embryoid body-derived cells (Figure 3-11A), cells derived from secondary blasts (Figure 3-11C) and D4T embryoid body-derived endothelial cells (included as a control; Figure 3-11D) with phosphorylation peaking at 2 minutes and declining to basal by 30 minutes. This VEGF-induced ERK1/2 phosphorylation profile is typical of ERK1/2 activation and correlates well with previous work in rat sinusoidal endothelial cells (Takahashi and Shibuya, 1997). An increase in ERK1/2 phosphorylation over basal was not visualised following 30 minute VEGF stimulation in contrast to findings reported by Park *et. al.* (Park et al., 2004) though in this previous study, extensive time-course experiments were not performed. This is possibly due to the comparatively higher background ERK1/2 phosphorylation observed in our study that may have been caused by culture of the embryoid bodies in serum containing medium. Park *et al.*, utilised serum-free culture conditions which may have decreased basal ERK1/2 phosphorylation allowing a greater window for visualisation of increases in ERK1/2 phosphorylation. Increasing the duration of serum-starvation of embryoid body-derived cells prior to VEGF-stimulation to 5 hours however, did not decrease basal ERK phosphorylation in this study.

3.4.3.3 Is VEGF- mediated ERK1/2 signalling activated via PLC γ ?

VEGF stimulation of day 5 primary embryoid body-derived cells caused a transient increase in PKC phosphorylation. Phosphorylation peaked at 2 minutes and was back to basal levels by 5 minutes correlating well with previous reports of VEGF-induced

PKC activation in bovine aortic endothelial cells (BAECs) in which VEGF stimulated formation of ^3H -DAG peaked by 5 minutes stimulation (Xia et al., 1996).

Work in HUVECs suggests that in adult human endothelial cells VEGF-induced MAPK activation occurs through a phospholipase C- γ (PLC γ)-dependent Protein Kinase C (PKC)-mediated mechanism rather than the classical Ras-Raf pathway (Doanes et al., 1999; Gliko et al., 2001; Yashima et al., 2001). However, further work by Yashima *et al.* demonstrated that the mechanism of VEGF activation of the MAPK pathway may be heterogenous between endothelial cells of different origins (Yashima et al., 2001). As VEGF-induced PKC phosphorylation occurred within the correct time frame that could allow PKC to mediate MAPK activation, the role of PKC in EB-derived cells was investigated further. Previous studies that have implicated VEGF-induced PLC γ 1 signalling through PKC activation, as a mechanism of MAPK activation, have relied heavily on the use of pharmacological inhibitors. PKC-mediated MAPK activation was demonstrated by Gliko *et al.*, and Doanes *et al.*, when inhibition of PKC with 1 μM Calphostin C also inhibited VEGF-induced activation of the MAPK pathway (Doanes et al., 1999; Gliko et al., 2001). Initial investigations were therefore carried out using the same conditions to ascertain whether this result would be replicated in embryoid body-derived cells. No inhibition of VEGF-stimulated ERK1/2 phosphorylation was observed when cells were preincubated with 1 μM Calphostin C. This suggests that VEGF-induced activation of the MAPK pathway in embryoid body-derived cells is not dependent on PKC.

Flk-1-mediated activation of the classical α and novel δ PKC isoforms has been implicated in experiments performed in human endothelial cells (Gliko et al., 2001; Kuriyama et al., 2004). Immunoblotting was therefore also performed with an anti-phospho-PKC δ antibody to investigate VEGF-induced PKC δ activation in primary embryoid body-derived cells. No increase in PKC δ phosphorylation was observed suggesting PKC δ may not be activated by VEGF stimulation in this system. Further investigations in which embryoid body-derived cells were pre-incubated with the classical PKC inhibitor Gö6796 prior to VEGF stimulation also demonstrated no inhibition of ERK1/2 phosphorylation at 30 or 300nM Gö6796, although some inhibition was seen at 3 μM Gö6796. Gliko *et al.*, used the inhibitor at 1 μM , but reported no such effect. As this experiment was only carried out once during the

course of my study, this finding is by no means conclusive. The reported IC_{50} of Gö6796 against isolated PKC α and β is 2.3nM (Martiny-Baron et al., 1993). It is therefore possible that any inhibition of VEGF stimulated ERK1/2 phosphorylation seen upon incubation with 3 μ M Gö6796 may have been due to non-specific inhibition or toxicity of the inhibitor.

The investigations conducted as part of this study suggest that PKC may not be an important mediator of VEGF-induced activation of the MAPK pathway in murine embryoid body-derived cells, a result also observed in Human Microvascular Endothelial Cells (HMVECs) (Yashima et al., 2001) thus this result is not without precedent. However, as the efficacy of PKC inhibition was not determined, additional, more detailed studies are required to confirm these observations. Possible further studies could include assessment of PKC inhibition using *in vitro* kinase assays. Alternatively, the effect of inhibition of PLC γ , with a pharmacological inhibitor such as U73122 (Bleasdale et al., 1989) on MAPK activation could be investigated and activation of PKC could be used to confirm PLC γ inhibition. If PKC/PLC γ involvement were demonstrated, the role of specific isoforms could be investigated in more detail using antibodies to detect phosphorylation of specific PKC isoforms upon VEGF-stimulation. Alternatively, incubation of embryoid body-derived ES cells with isoform specific inhibitors such as rottlerin, followed by VEGF stimulation and immunoblotting with the pan-phospho-PKC antibody, or, siRNA knockdown of specific PKC isoforms in Flk-1 expressing ES cells (see chapter 5), again monitored by immunoblotting with a pan-phospho-PKC antibody, could be used to investigate the isoform specificity of VEGF-stimulated PKC activation.

3.4.3.4 VEGF does not appear to activate PI3K in primary embryoid body-derived cells

VEGF stimulation of primary embryoid body-derived cells, secondary blast-derived cells and the D4T embryoid body-derived endothelial cell line failed to demonstrate any increase in phosphorylation in a number of downstream effectors of PI3K signalling. However, VEGF stimulation of HUVEC cells with the same concentration of murine VEGF over the same time-course did demonstrate

phosphorylation of downstream effectors of the PI3K signalling pathway suggesting the time-course and ligand concentration used were not the cause of this apparent lack of activation. There are a number of possibilities to explain these results. Firstly,

it is possible that VEGF does induce PI3K activation but to only a small degree such that the increase in phosphorylation of downstream effectors is small compared to basal levels of PI3K activation and therefore difficult to visualise by immunoblotting. The EB-derived cells and D4T cells exhibited significant phosphorylation of downstream effectors, even under non-treated conditions (see Figure 3-11 A,C and D) indicative of high basal levels of PI3K activity. The high basal levels of PI3K activity could mask any potential stimulatory effect of VEGF on further activation of this pathway. If this is the case elucidation of any VEGF-induced contribution to cellular PI3K activity would be difficult. High basal levels of PI3K activity were also seen in VEGF-stimulated Flk-1 null embryoid body-derived cells suggesting that the high levels of basal PI3K activation were not mediated through the Flk-1 receptor. Flk-1 mediated PI3K activation could be investigated further by measurement of D3 phosphoinositides within the cells, under basal conditions and following VEGF stimulation. The interaction of PI3Ks with Flk-1 could also be investigated using Flk-1 immunoprecipitation and immunoblot analysis with anti-p85 and anti-Shb antibodies.

If the PI3K pathway is indeed not activated by VEGF stimulation in embryoid body-derived cells, as the data generated during this study suggests, this could indicate that there are significant differences in the requirements for Flk-1 signalling between adult and developmental systems. In human endothelial cells VEGF-induced PI3K activation is important for a number of processes including proliferation (Dayanir et al., 2001; Hamada et al., 2005), migration (Holmqvist et al., 2004), cell survival (Fujio and Walsh, 1999; Gerber et al., 1998) and eNOS production (Bates and Harper, 2002; Fulton et al., 1999; Lin and Sessa, 2006). The requirements of Flk-1 signalling during early development and in the adult are somewhat different. For example, the eNOS knockout mouse shows no remarkable phenotype demonstrating only a limb defect unrelated to the development of haemopoietic and endothelial cells (Gregg et al., 1998) whilst in adult endothelial cells eNOS production is an important downstream effect of Flk-1 signalling, mediating vascular smooth muscle

relaxation. Important requirements for Flk-1 signalling during haemopoietic and endothelial differentiation are likely to include migration, proliferation, differentiation and cell survival. Proliferative and differentiative signals can be transduced through the MAPK pathway which is demonstrably activated upon VEGF stimulation (see Figure 3-8). Furthermore, it is possible that cell survival in EB-derived cells is mediated by other signalling pathways.

A recent study into the importance of PI3K signalling in early development and haemopoiesis found that inhibition of the PI3K pathway, pharmacologically using the LY294002 inhibitor, or genetically using a PDK-1^{-/-} ES cell line, caused reduced cellularity upon embryoid body formation. This reduced cellularity was due to a defect in proliferation, rather than an increase in cellular apoptosis. Furthermore, inhibition of the PI3K pathway also inhibited expansion of blast colonies. However, the formation of the BL-CFC and its differentiation towards the haemopoietic lineage were not affected and differentiation towards the haemopoietic lineage was actually enhanced (Bone and Welham, 2007). It may be the case that although the PI3K pathway is important during development it does not play a role downstream of Flk-1 signalling during early embryoid body development. Basal levels of phosphorylation of the down-stream mediators of PI3K probed for in the immunoblots of VEGF-stimulated primary embryoid body-derived cells were high, even in Flk-1^{-/-} EBs, suggesting activation of this pathway, even during serum-starvation, is not mediated by Flk-1 dependent signalling (e.g through a VEGF autocrine loop). Further studies using pharmacological inhibitors such as LY294002 and wortmannin to gauge the importance of PI3K signalling in VEGF/Flk-1 mediated processes such as endothelial differentiation may help to elucidate its role (see section 4.2.1.).

3.4.3.5 Effects of inhibiting the PI3K and MAPK pathways on VEGF-induced MAPK activation

Inhibition of the MAPK pathway with the MEK1/2 inhibitor U0126 caused inhibition of all ERK1/2 phosphorylation but had no effect on the phosphorylation status of the S6 ribosomal protein, a downstream effector of the PI3K pathway. Inhibition of PI3K with LY294002 not only inhibited phosphorylation of S6

ribosomal protein, but also increased phosphorylation of ERK1/2, suggesting that the PI3K pathway inhibits activation of the MAPK pathway in primary embryoid body-derived cells. ERK1/2 phosphorylation increased not only in the VEGF-stimulated samples but also, perhaps to a lesser extent, in the unstimulated control. Increased basal ERK1/2 phosphorylation upon pharmacological and genetic inhibition of the PI3K pathway was also observed in undifferentiated ES cells by Paling *et al.*, (Paling *et al.*, 2004). The results presented here provide evidence of cross-talk between the MAPK and PI3K pathways in primary embryoid body-derived cells and correlate well with work performed by Blum *et al.*, demonstrating an inhibitory role for PI3K in VEGF induced MAPK-dependent tissue factor expression (Blum *et al.*, 2001). PI3K was also found to inhibit EGF-induced MAPK signalling in HEK293 cells and studies into the mechanism of PI3K inhibition of MAPK signalling in this system revealed that the cross-talk between the PI3K and MAPK signalling pathways may be mediated by PKB phosphorylation and inactivation of Raf, an upstream effector of MAPK signalling in many cell types (Zimmermann and Moelling, 1999). More recently, a study in zebrafish has implicated opposing roles of VEGF-induced MAPK activation and PI3K activation (which the authors also considered to be VEGF-induced) as important for endothelial specification towards either an arterial (MAPK) or venous (PI3K) fate (Hong *et al.*, 2006).

As no VEGF-induced phosphorylation of downstream effectors of the PI3K signalling cascade was observed in primary embryoid body-derived cells it is possible that this pathway is not activated downstream of Flk-1 until later in development or in the adult. If this is the case, then it raises questions as to how Flk-1-mediated PI3K signalling is either initially depressed or subsequently up-regulated. It is possible that the adapter protein/s which later couple Flk-1 to PI3K are not expressed in Flk-1 positive cells at this time. To investigate this possibility, Flk-1 immunoprecipitations could be probed with antibodies raised against candidate adapter proteins or expression of adapter protein transcripts in embryoid body-derived cells sorted for Flk-1 expression could be assessed using RT-PCR. Potential candidate adaptor proteins linking Flk-1 to PI3K include Gab1, Shb and VRAP/TSAd (see section 1.4.2). Of these Shb is required for mesodermal formation so is likely to be expressed in conjunction with Flk-1 (Kriz *et al.*, 2006; Rolny *et al.*, 2005) and Gab1 is expressed during this stage of embryogenesis, though in neither

case is their localisation within the embryo reported (<http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Mm.277409> accessed 7th September, 2007). VRAP/TsAd expression, however, has not been reported at this early stage of embryoid body formation (<http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Mm.86361> accessed 7th September 2007) though it has been noted in some blood vessels of the day 8 embryoid body by Matsumoto *et al.*, (Matsumoto et al., 2005).

3.4.4 Conclusions

E14tg2A embryonic stem cells can be differentiated to form primary embryoid bodies as an *in vitro* model of embryogenesis. Cells from dissociated embryoid bodies express the receptor tyrosine kinase Flk-1 from day 3.5 of differentiation, with expression peaking between days 4 and 6 and declining thereafter. VEGF stimulation of these embryoid body-derived cells results in transphosphorylation of the receptor itself and subsequent activation of the MAPK and PLC γ 1 signalling pathways. However, VEGF does not appear to activate the PI3K pathway in these cells, despite VEGF-mediated PI3K signalling playing an important role in adult endothelial cells. However, inhibition of the PI3K cascade increases ERK phosphorylation suggesting cross-talk between these two pathways. A summary of VEGF mediated Flk-1 signalling elucidated so far is shown in Figure 3-13.

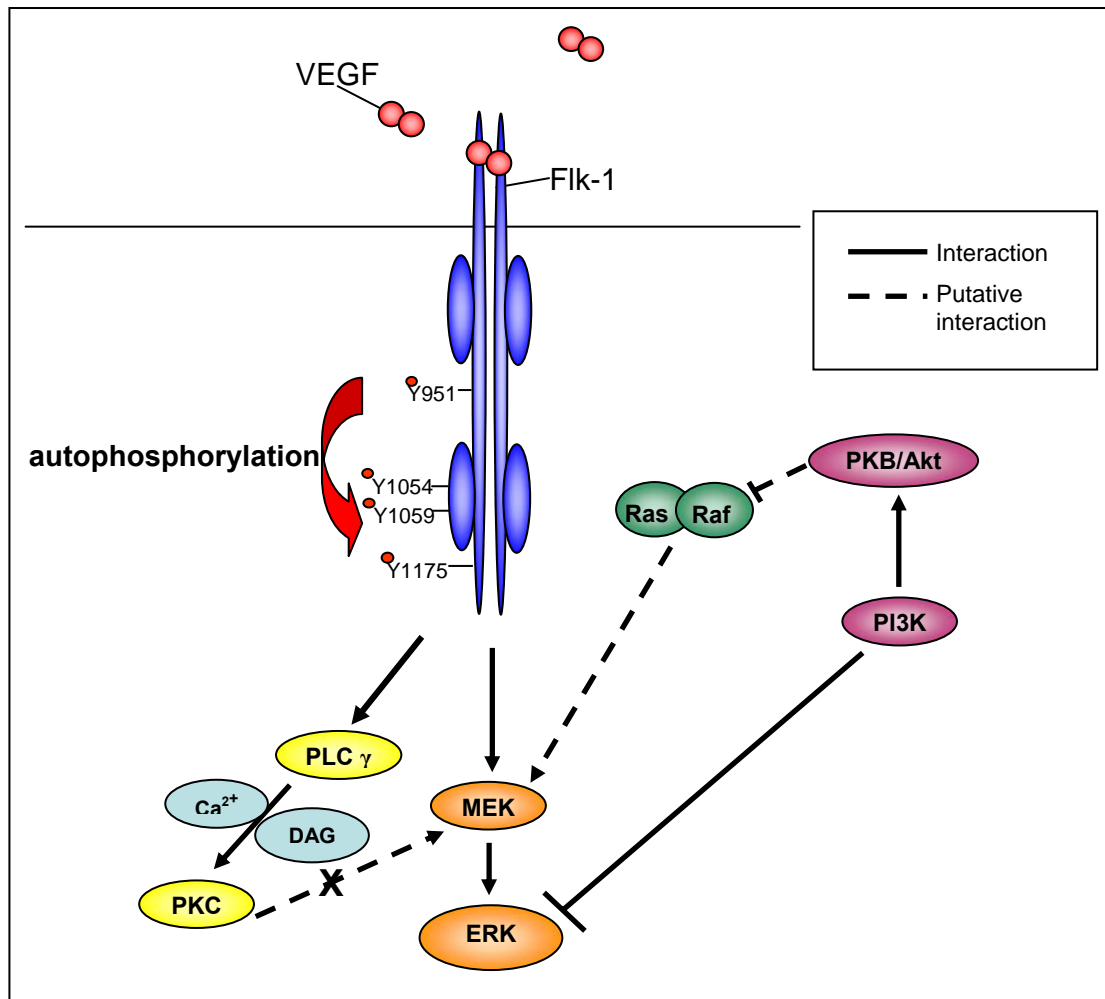


Figure 3-13 Model of VEGF-induced Flk-1 signalling in early embryoid body development based upon signalling studies in chapter 3.

Chapter 4

The role of Flk-1

signalling during ES cell

differentiation towards

endothelial and

cardiomyocyte lineages

4.1 Introduction

In chapter 3 I discovered that VEGF stimulation of Flk-1 in an embryoid body model of development led to activation of the MAPK and PLC γ 1 pathways but, unlike in adult endothelial cells, not the PI3K pathway. To further elucidate the importance of Flk-1-mediated signalling during *in vitro* models of development functional assays were performed to investigate the importance of Flk-1 signalling during ES cell differentiation along endothelial and cardiac lineages.

Early research into the role of Flk-1 during development, demonstrated its role in endothelial cell specification. The Flk-1 knockout mouse dies due to a failure in vasculogenesis (Shalaby et al., 1995) and it has recently been demonstrated that all early embryonic endothelial cells arise from Flk-1 positive progenitors (Ueno and Weissman, 2006). The role of Flk-1 in the development of cardiac lineages has been discovered more recently. *In vivo* lineage tracing experiments followed Flk-1 positive progenitors exiting the primitive streak to the cardiac crescent (Ema et al., 2006). *In vitro* models of cardiogenesis have demonstrated that VEGF treatment up-regulates cardiac markers whilst treatment with an anti-Flk-1 antibody decreases their expression (Chen et al., 2006). Furthermore, the Keller group identified a second cell population which up-regulated Flk-1 later than cells fated to the haemangioblast from which cardiac precursors arose (Kattman et al., 2006).

The temporal requirement for Flk-1 expression and the downstream signals involved in cardiac and endothelial specification, vasculogenesis and angiogenesis in the embryo have not yet been elucidated. In this chapter *in vitro* models of vasculogenesis/angiogenesis and the formation of beating cardiomyocytes were used alongside Flk-1 knockout ES cells and pharmacological inhibitors of Flk-1, the MAPK pathway and the PI3K pathway to investigate the importance of the timing of Flk-1 expression and its downstream signals during differentiation towards the endothelial and cardiomyocyte lineages.

4.2 The role of Flk-1 and its downstream signalling in endothelial development

The first step in the generation of a functional circulatory system is the formation of a primitive vascular plexus in which nascent vessels consist of a tube of endothelial cells (Jain, 2003). Flk-1 is expressed on the early endothelial precursors which differentiate into the endothelial cells of these early vessels (Ueno and Weissman, 2006) and the Flk-1 null animal dies due to a failure in formation of such blood vessels (Shalaby et al., 1995). In the adult animal Flk-1 is required for angiogenesis and its expression is maintained on adult endothelial cells (Zachary, 2001). The precise role of Flk-1 in early vessel formation has been difficult to study due to the early embryonic lethality of Flk-1 null mice. A migratory role has been implicated for Flk-1 in guiding endothelial precursors (Shalaby et al., 1997) and *in vitro* work, using Flk-1 null ES cells, reported formation of endothelial precursors in an embryoid body system, (Schuh et al., 1999) supporting the hypothesis that endothelial precursors can be specified in the absence of Flk-1 but fail to migrate *in vivo* causing the lethal phenotype.

To investigate the importance and possible function of Flk-1 and its downstream signalling in embryonic vasculogenesis and angiogenesis, an embryoid body-based endothelial sprouting assay from Stem Cell Technologies was used. The assay was performed in two stages, embryoid body formation, during which vasculogenesis occurred (endothelial specification) followed by transplantation of the embryoid bodies into a collagen matrix, in which sprouting angiogenesis occurred (angiogenic expansion). This allowed dissection of the role of Flk-1 (using Flk-1^{-/-} ES cells and the Flk-1 inhibitors, VEGFR2 kinase inhibitors I and II) and the MAPK and PI3K pathways (using their inhibitors U0126 and LY294002 respectively) in both processes.

The basis of the Stem Cell Technologies endothelial sprouting assay was a procedure developed by Feraud *et al.*, as a method of studying early vasculogenesis and angiogenesis processes which are relatively inaccessible for study in the embryo (Feraud et al., 2001). Earlier studies had demonstrated formation of extensive

vascular structures in a majority of embryoid bodies differentiated for 10-11 days (Hirashima et al., 1999; Risau et al., 1988; Vittet et al., 1996; Wang et al., 1992). Feraud *et. al.* demonstrated that when these embryoid bodies were placed in a matrix of type I collagen (a major component of pericapillary connective tissue), in the presence of angiogenic growth factors, endothelial cells migrated out and formed visible projections in a process of sprouting angiogenesis. Pecam1 and vWF marker expression demonstrated that whilst some cellular projections were not attributable to the endothelial lineage ~50% of the sprouts were true endothelial sprouts. Embryoid bodies cultured in the sprouting phase in the absence of angiogenic cytokines were unable to produce sprouts of an endothelial origin. Removal of VEGF from the angiogenic cytokine cocktail reduced the number of endothelial sprouts by 80% indicating that VEGF signalling plays an important role in the formation of angiogenic sprouts in the assay. However, the addition of the growth factor cocktail also increased the number of non-endothelial sprouts indicating pleiotrophic effects (Feraud et al., 2001).

Upon conducting the sprouting angiogenesis assay it became apparent that the extent of sprouting of embryoid bodies under different conditions must be taken into account in order for true comparisons between treatments to be made. To this end three definitions describing the extent of sprouting from embryoid bodies were used. These were many, few or no sprouts, defined as follows, and illustrated in Figure 4-1:

Many sprouts defined embryoid bodies from which more than 5 visible sprouts of greater than the radius of the embryoid body or more than 10 visible sprouts of less than the radius of the embryoid body were projected. Embryoid bodies exhibiting ‘many’ sprouts were considered likely to represent angiogenic sprouting.

Few sprouts defined embryoid bodies which formed less sprouts than would fit the parameters of ‘many’. This degree of sprouting was observed in embryoid bodies cultured in the absence of angiogenic cytokines which are reportedly unable to produce endothelial sprouts (Feraud et al., 2001). They were therefore considered most likely ‘non-endothelial’.

No sprouts defined embryoid bodies which formed no sprouting projections in the sprouting angiogenesis assay.

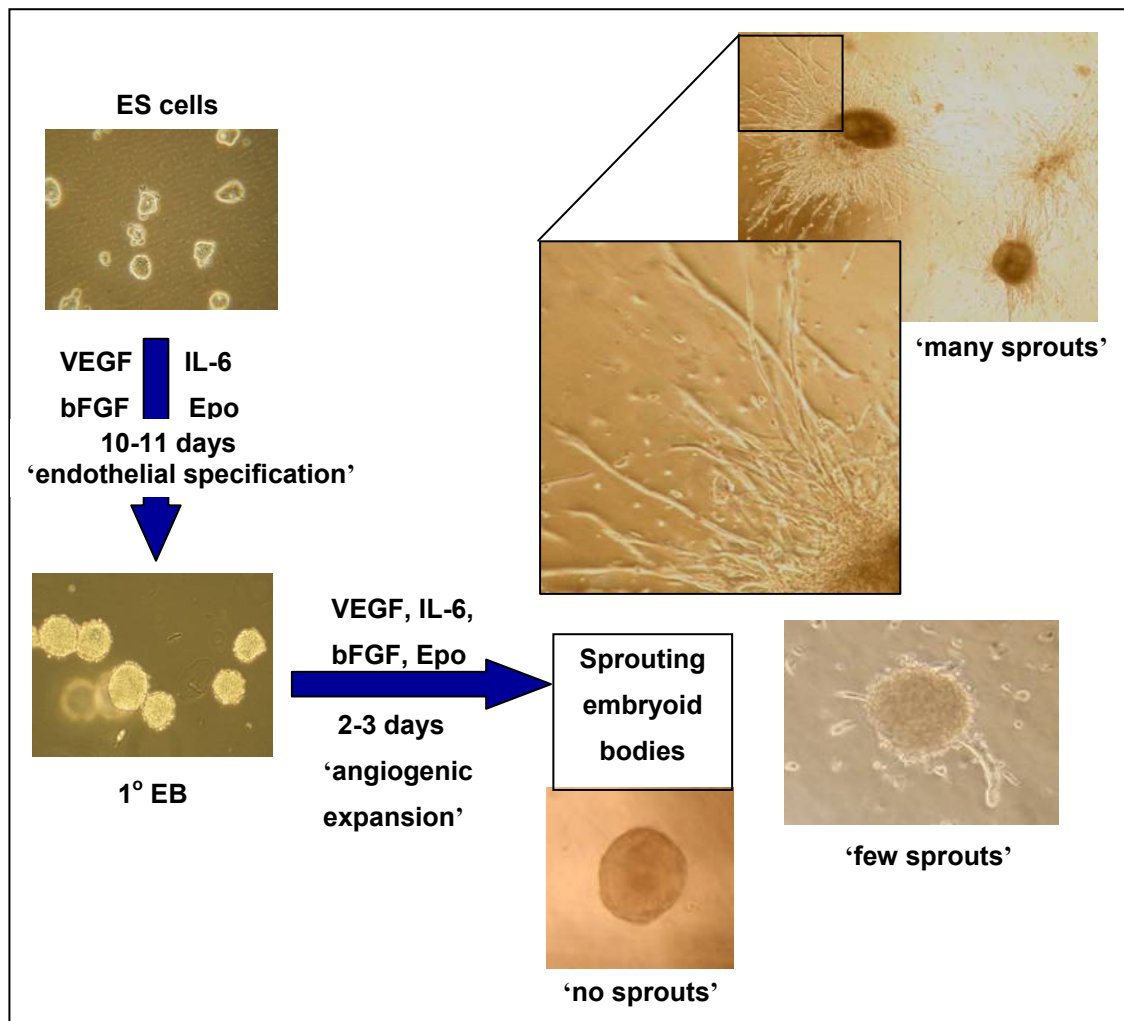


Figure 4-1 The endothelial sprouting assay. Embryonic stem cells were differentiated in methylcellulose in the presence of angiogenic cytokines. After 10-11 days they were replated into fresh endothelial medium containing a collagen matrix. Sprouting, indicative of sprouting angiogenesis, was visible from 2 days onwards. Embryoid bodies under different conditions produced many, few or no sprouts (for definitions see previous text). The extent of sprouting under different experimental conditions was quantified in duplicate for each independent experiment.

4.2.1 Effects of inhibition or deletion of Flk-1 on endothelial sprouting

Initial characterisation of the role or requirement for Flk-1 in the endothelial sprouting assay was required before the importance of various downstream signalling pathways could be dissected. For this a Flk-1 knockout ES cell line and two structurally distinct pharmacological inhibitors of Flk-1, VEGFR2 kinase inhibitors I and II were utilised. The use of inhibitors allowed dissection of the role of Flk-1 in one or both stages of the assay and allowed validation of results gained using the Flk-1 knockout ES cell line.

4.2.1.1 Dose optimisation of Flk-1 inhibitors

Two Flk-1 inhibitors were used in the study; VEGFR2 kinase inhibitor-1 ((z)-3[(2,4-dimethyl-3(ethoxycarbonyl)pyrrol-5-yl)methylidenyl]indolin-2-one; termed KI1) (Sun et al., 1998) and VEGFR2 kinase inhibitor-2 ((z)-5-Bromo-3-(4,5,6,7-tetrahydro-1H-indol-2-methylene)-1,3-dihydroindol-2-one; termed KI2) (Sun et al., 2000). The mechanism of action of both was competitive inhibition at the ATP binding site. As the Flk-1 inhibitors had not been previously characterised in an ES cell system dose optimisation for Flk-1 inhibition was required. The reported IC_{50} of KI1 for inhibition of tyrosine kinase activity in an NIH3T3 cell line engineered to overexpress Flk-1 was 70nM. The compound was quoted as having >1000-fold selectivity over PDGFR β , EGF-R and IGF-1R receptor tyrosine kinases (RTKs) (Sun et al., 1998). The reported IC_{50} of KI2 against Flk-1 in a peptide-based transphosphorylation assay was also 70nM though KI2 was less selective with regard to PDGF-R β for which it's reported IC_{50} (this time in a biochemical tyrosine autophosphorylation assay) was 920nM. IC_{50} 's for inhibition of ligand-induced cell proliferation, measured by incorporation of BrdU, were 110nM for inhibition of VEGF-induced HUVEC proliferation and 2.11 μ M for PDGF-induced proliferation of NIH-3T3 cells (Sun et al., 2000). As the assays used to measure IC_{50} values for Flk-1 and PDGF β R were not identical it is difficult to compare the IC_{50} values obtained. However, the possibility of non-selective inhibition of PDGF β R needs to be taken into account when using KI2.

As optimisation of specific antibodies detecting tyrosine phosphorylated residues on Flk-1 in our immunoblotting system had not been possible Flk-1-mediated ERK1/2 phosphorylation was used as a measure of Flk-1 activation status. Day 5 embryoid bodies were dissociated and the dissociated cells obtained were serum starved for 3 hours prior to a 30 minute preincubation with the inhibitor or DMSO as a control. Cells were then stimulated with 20ng/ml VEGF for 0, 2 or 10 minutes.

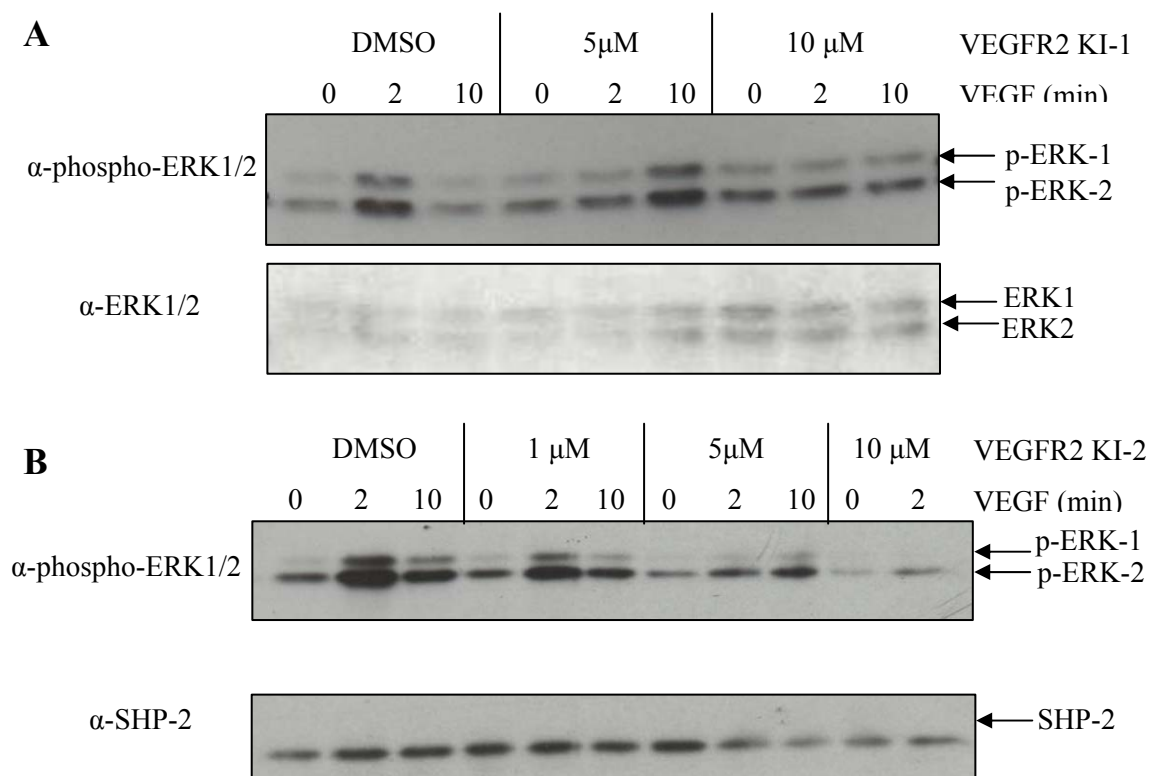


Figure 4-2 VEGF-stimulated primary embryoid body-derived cells pretreated with A) VEGFR2 kinase inhibitor-1 and B) VEGFR2 kinase inhibitor-2. Dissociated day 5 embryoid body-derived cells were serum starved for 3 hours prior to a 30 minute incubation with a DMSO control, VEGFR2 kinase inhibitor 1 (KI-1) or VEGFR2 kinase inhibitor-2 (KI-2). Cells were then stimulated with 20ng/ml VEGF for 0, 2 or 10 minutes. Immunoblotting was performed and blots were probed with an anti-phospho ERK1/2 antibody and then stripped and reprobed with anti-ERK1/2 and anti-SHP-2 antibodies to check for equal loading. The blots shown are representative of 2 individual experiments. 500nM VEGFR2 kinase inhibitor-1 was also used but this did not diminish ERK phosphorylation.

Figure 4-2 illustrates that 5 μ M of both VEGFR2 inhibitors appears to partially inhibit Flk-1-induced ERK1/2 phosphorylation, possibly shifting the time course of Flk-1-mediated ERK1/2 activation which appears to peak at 10 minutes rather than 2 minutes. Although 10 μ M of both inhibitors appears to facilitate greater inhibition of ERK1/2 phosphorylation inhibition with 10 μ M KI-2, also appears to inhibit basal ERK phosphorylation suggesting off-target inhibition of other signalling pathways that activate MAPK signalling. Thus, 5 μ M of both inhibitors was used in the functional endothelial sprouting assay.

4.2.1.2 Importance of Flk-1 for endothelial sprouting in primary embryoid bodies

Flk-1 is required for vasculogenesis and angiogenesis *in vivo* (Jain, 2003; Shalaby et al., 1995). To validate the endothelial sprouting assay as a model for the study of the involvement of Flk-1 and Flk-1 signalling during endothelial development, molecular and genetic approaches were used to characterise its importance in the endothelial sprouting assay.

Firstly, the ability of embryoid bodies derived from Flk-1^{-/-} ES cells or R1 parental ES cells to form angiogenic sprouts was assessed. As illustrated by Figure 4-3 in the presence of cytokines, some Flk-1^{-/-} embryoid bodies did produce ‘many’ sprouts though significantly fewer than the percentage of wild type R1 embryoid bodies. This suggests that either the Flk-1^{-/-} EBs contained less angiogenic precursors, that the sprouts from these embryoid bodies were not of endothelial origin or that expansion of all angiogenic precursors was not possible.

Sprouting was reduced in the absence of angiogenic cytokines, as previously reported (Feraud et al., 2001). There was no significant difference between the number of sprouting Flk-1^{-/-} embryoid bodies compared with R1 embryoid bodies in the absence of cytokines.

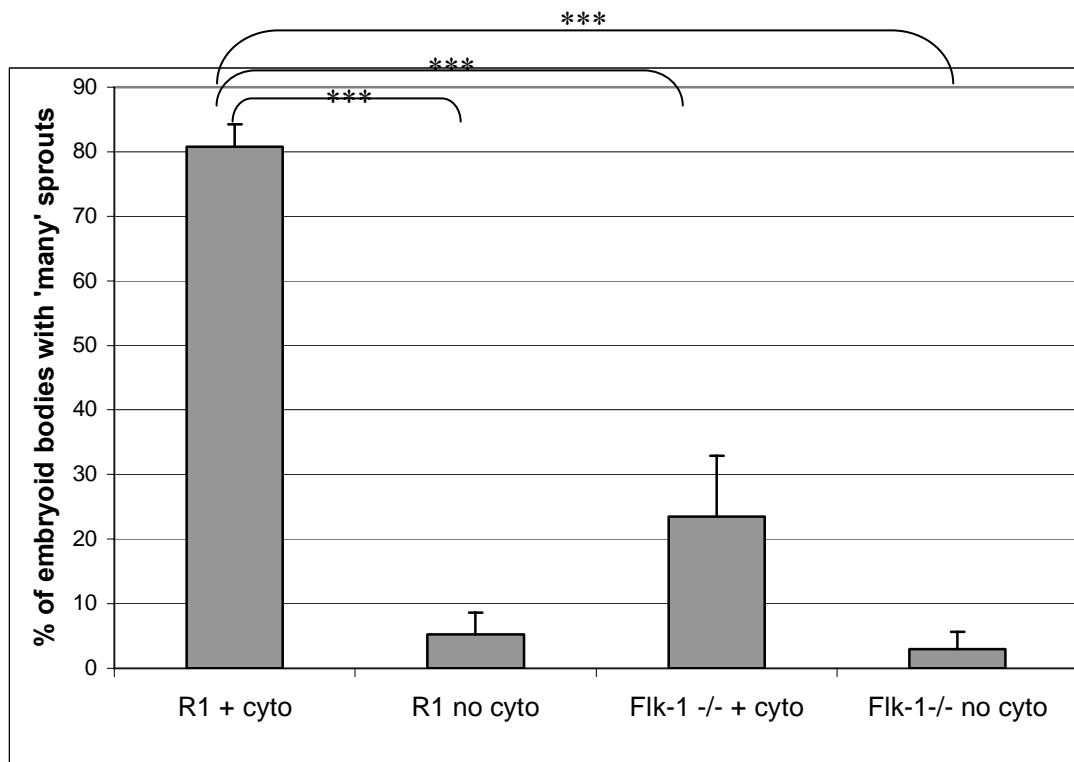


Figure 4-3 Comparison of sprout formation between R1 and Flk-1^{-/-} primary embryoid bodies. R1 and Flk-1^{-/-} primary embryoid bodies were cultured with angiogenic cytokines for 10 days prior to being placed in a collagen matrix, with (+cyto) or without (no cyto) angiogenic cytokines, for 2 days to promote sprouting indicative of angiogenesis. The percentage of embryoid bodies sprouting was quantified using the parameters set out in section 4.2. Results shown represent pooled data from 6 experiments. The chart illustrates the percentage of embryoid bodies from each culture condition which exhibited 'many' sprouts indicative of sprouting angiogenesis. Error bars represent s.e.m. ANOVA demonstrated significant differences between the four culture conditions which were analysed further using Tukeys honestly significant difference post-hoc test. Significant differences are illustrated *** $p < 0.01$.

To confirm that the reduction in sprouting observed with Flk-1 null EBs was due to the lack of Flk-1 expression, rather than other differences between the cell lines, R1 wild type EBs in the assay were incubated with the Flk-1 inhibitors VEGFR2 kinase inhibitor-1 (KI-1) and VEGFR2 kinase inhibitor-2 (KI-2). Additionally, treatment with KI-1 or KI-2 in the first stage of embryoid body formation, the second stage of angiogenic sprouting or both stages of the assay allowed investigation of the relative importance of Flk-1 during each of the two phases of the assay, which represent endothelial specification and angiogenic expansion respectively. The results are

summarised in Figure 4-4 (KI-1 treatment) and 4-5 (KI-2 treatment). Both inhibitors generally decreased the extent of sprouting. Treatment with 5 μ M KI-1 (illustrated in Figure 4-4) in either the first or both phases of the assay significantly decreased the number of embryoid bodies exhibiting ‘many’ sprouts though not to the extent observed in Flk-1^{-/-} embryoid bodies in the same experiments. Treatment with 5 μ M KI-1 in the second phase of the assay also appeared to decrease the number of embryoid bodies with ‘many’ sprouts though this decrease was not statistically significant. This decrease in sprouting supports the data from Flk-1^{-/-} embryoid bodies though the decrease in sprouting was less dramatic. This may be due to incomplete inhibition of Flk-1 or instability of the inhibitor in a long-term assay.

Treatment of embryoid bodies during both the first phase of development and in the second sprouting phase with 5 μ M VEGFR2 kinase inhibitor-2 significantly inhibited their ability to sprout (Figure 4-5). Inhibition of Flk-1 with KI-2 in both phases appeared to further decrease sprouting when compared with inhibition in either the first or second phase suggesting an additive mechanism. Treatment with 500nM KI-2 showed a similar pattern of inhibition of sprouting though to a lesser extent in the first phase. As this experiment was only performed once it is unclear whether this inhibition was significant. The KI-2 data supports data previously obtained using Flk-1^{-/-} embryoid bodies (Figure 4-3) and validates the assay as a mechanism for studying the involvement of Flk-1 signalling in vasculogenesis and angiogenesis. Comparing results obtained using either KI-1 or KI-2 it is clear that KI-2 had a greater inhibitory effect on endothelial sprouting. This could be due to incomplete inhibition by KI-1, the possibility of off-target effects of KI-2 or a combination of both factors.

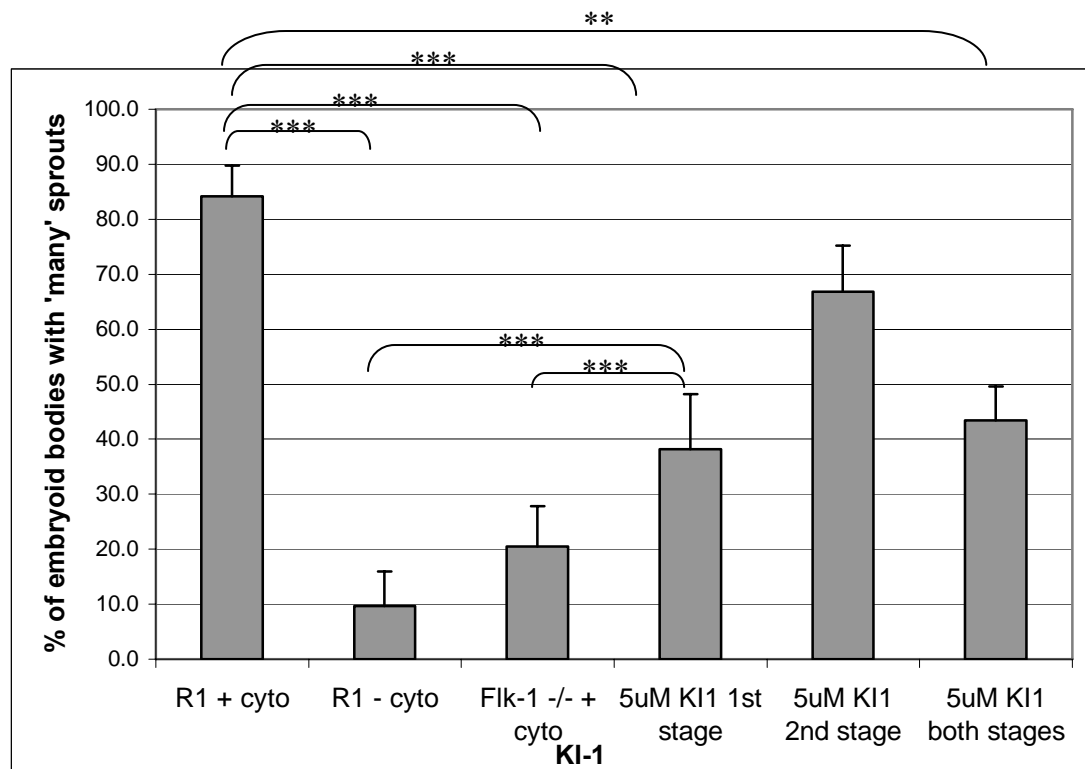


Figure 4-4 Comparison of sprout formation between R1 and Flk-1/- primary embryoid bodies and those treated with the Flk-1 inhibitor VEGFR2 kinase inhibitor-1 (KI-1) Primary embryoid bodies were cultured with angiogenic cytokines and in the presence or absence of KI-1 for 10 days (1st stage) prior to being placed in a collagen matrix, with or without KI-1, for a further 2 days (2nd stage) to enable sprouting indicative of angiogenesis to proceed. The percentage of embryoid bodies sprouting was quantified using the parameters set out in section 4.2. Figure 4-4 represents pooled data from 3 independent experiments in which embryoid bodies were treated in the first, second or both stages of the assay (as indicated) with 5 μ M KI-1. The graph illustrates the % of embryoid bodies from each culture condition that exhibited 'many' sprouts indicative of angiogenic sprouting. Error bars represent s.e.m. ANOVA showed significant differences in sprouting between different culture conditions which were further analysed using Tukeys honestly significantly different post-hoc test. Significant differences, where shown, are illustrated as follows ** $p < 0.05$, *** $p < 0.01$.

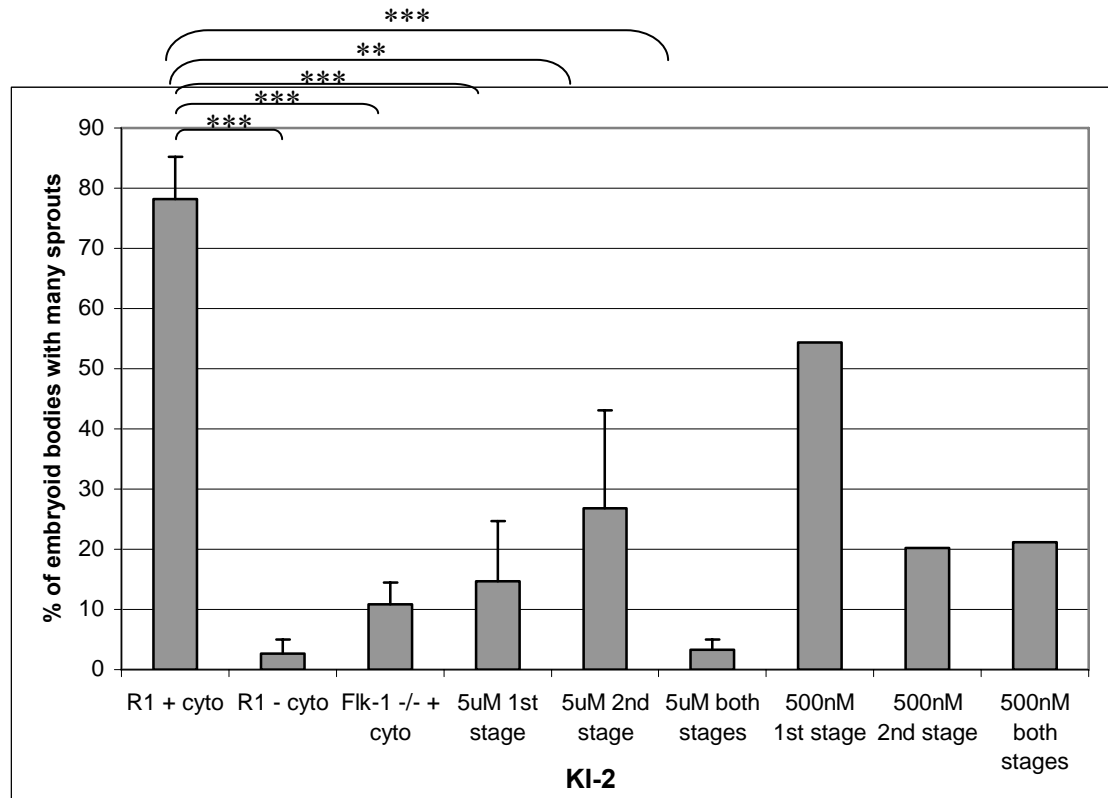


Figure 4-5 Comparison of sprout formation between R1 and Flk-1/- primary embryoid bodies and those treated with the Flk-1 inhibitor VEGFR2 kinase inhibitor-2 (KI-2) Primary embryoid bodies were cultured with angiogenic cytokines and in the presence or absence of KI-2 for 10 days (1st stage) prior to being placed in a collagen matrix, with or without KI-2, for a further 2 days to enable sprouting indicative of angiogenesis to proceed.. The percentage of embryoid bodies sprouting was quantified using the parameters set out in section 4.2. The graph represents pooled data from 3 independent experiments using 5µM KI-2, and one independent experiment in which EBs were incubated with 500nM KI-2, in which embryoid bodies were treated in the first, second or both stages of the assay (as indicated) with KI-2. The percentage of embryoid bodies with ‘many’ sprouts, indicative of angiogenic sprouting from each culture condition is illustrated. Error bars represent s.e.m. ANOVA found significant differences between treatments. These differences were further analysed using Tukey’s honestly significantly different post-hoc test. Significant differences, where shown, are illustrated as follows *** $p < 0.01$, ** $p < 0.05$

4.2.1.3 The importance of VEGF in endothelial sprouting from primary embryoid bodies

The absence of VEGF from the second phase of the endothelial sprouting assay reportedly decreases sprouting by up to 80% (Feraud et al., 2001). To confirm this and assess its importance as an extrinsic cytokine in both stages of the assay the assay was performed using R1-derived embryoid bodies in the presence and absence of extrinsic VEGF for both stages.

Figure 4-6 demonstrates that the absence of extrinsic VEGF significantly decreased sprouting by R1 embryoid bodies in both the first and second phases confirming preliminary work published by Feraud *et. al.* upon development of the assay (Feraud et al., 2001). The level of reduction in the percentage of embryoid bodies with ‘many’ sprouts was similar to the 80% previously when extrinsic VEGF was omitted in the second phase, a greater reduction than observed in its absence from the first phase, although this was not significant. This correlates well with data from Ng *et al.*, using VEGF null ES cells. No requirement for VEGF was found during endothelial specification but VEGF was required for vessel formation (Ng et al., 2004). As VEGF is reportedly required for vessel formation the extent of sprouting in embryoid bodies cultured in the absence of exogenous VEGF suggests that VEGF is being secreted by cells within the embryoid bodies. Semi-quantitative RT-PCR using RNA extracted from day 11 embryoid bodies demonstrated the presence of VEGF mRNA, confirming its production by the embryoid bodies themselves.

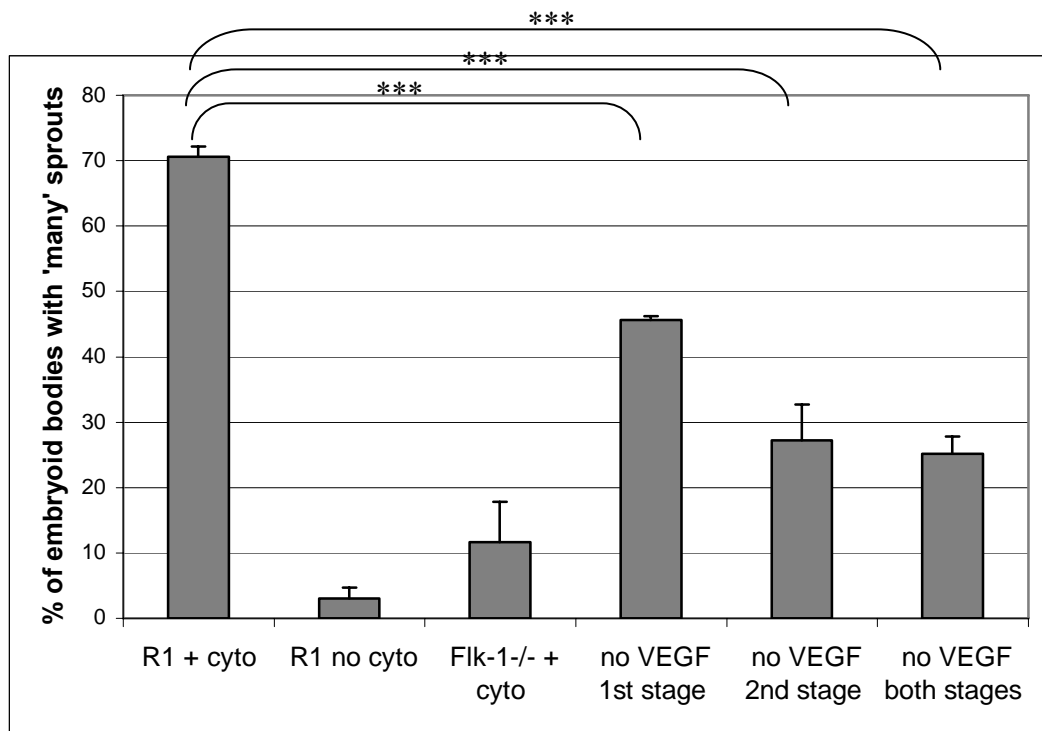


Figure 4-6 Comparison of sprout formation in the presence and absence of extrinsic VEGF.

R1 primary embryoid bodies were cultured for 10 days prior to being placed in a collagen matrix for 2 days to promote sprouting indicative of angiogenesis. In both stages embryoid bodies were cultured in the presence or absence of a cytokine cocktail within which the cytokine VEGF was itself present or absent as indicated. The percentage of embryoid bodies sprouting was quantified using the parameters set out in section 4.2. Results shown represent pooled data from 3 independent experiments illustrating the % of embryoid bodies from each culture condition which exhibited 'many' sprouts. Error bars represent s.e.m. ANOVA demonstrated significant differences between the five culture conditions that were analysed further using Fisher's least significant difference post-hoc test. Significant differences, were illustrated *** $p \leq 0.01$

4.2.2 Effect of PI3K inhibition on endothelial sprouting

Work discussed in chapter 3 (see section 3.3.4) demonstrated no apparent activation of PI3K upon VEGF stimulation of Flk-1 in cells derived from primary embryoid bodies. This contrasts with previous work in adult endothelial cells (Dayanir et al., 2001; Gerber et al., 1998). In the adult organism PI3K activation promotes angiogenesis, at least in part by up-regulating expression of VEGF (Jiang et al., 2000) making it a popular target in anti-cancer therapeutics (Hennessy et al., 2005). PI3K signalling *downstream* of VEGF/Flk-1 signalling also contributes to angiogenesis, promoting endothelial cell migration via an eNOS-dependent

mechanism (Morales-Ruiz et al., 2000). Moreover, recent work using embryoid bodies as a model of early development has cited involvement of the PI3K and MAPK pathways in the divergence of arterial and venous cell fates respectively (Hong et al., 2006). As Flk-1 does not appear to mediate PI3K activation in primary embryoid bodies, LY294002, a broad range PI3K inhibitor was used to investigate whether PI3K is involved in angiogenic sprouting in the endothelial sprouting assay. Previous work had demonstrated that complete inhibition of the PI3K pathway using either 10 μ M LY294002 or a PDK-1^{-/-} ES cell line impairs embryoid body formation (Bone and Welham, 2007) meaning that only a partial inhibition with 5 μ M LY294002 could be used to investigate the role of this family of kinases in endothelial sprouting.

Figure 4-7 illustrates that neither the inclusion of 5 μ M LY294002 in the assay nor the inclusion of 10 μ M LY294002 in the second, angiogenic expansion phase of the assay affected the percentage of embryoid bodies which exhibited ‘many’ sprouts. Partial inhibition of this pathway therefore does not appear to affect endothelial specification or angiogenic expansion in an embryoid body model of developmental vasculogenesis and angiogenesis. This supports earlier results (see section 3.3.4) in which activation of Flk-1, itself an important mediator of endothelial specification and angiogenic expansion, did not appear to mediate downstream PI3K signalling.

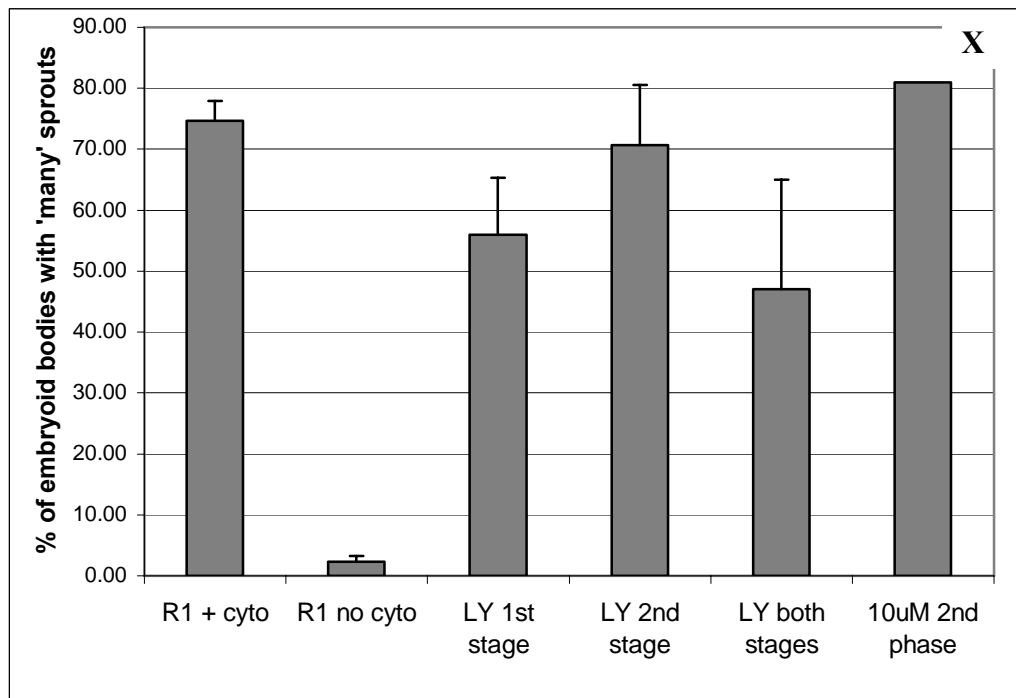


Figure 4-7 Comparison of sprout formation in the presence and absence of the PI3K inhibitor LY294002. R1 primary embryoid bodies were cultured for 10 days prior to being placed in a collagen matrix for 2 days to promote sprouting indicative of angiogenesis. In both stages embryoid bodies were cultured in the presence or absence of a cytokine cocktail. The percentage of embryoid bodies sprouting was quantified using the parameters set out in section 4.2. Results shown represent pooled data from 3 independent experiments (with the exception of 10uM LY294002 (denoted X) which is representative of 2 independent experiments). The % of embryoid bodies from each culture condition which exhibited 'many' sprouts, indicative of angiogenic sprouting, is illustrated. Error bars represent s.e.m. ANOVA demonstrated no significant differences in the percentage of embryoid bodies with 'many' sprouts between the cytokine treated embryoid bodies in the DMSO control and those cultured in the presence of 5 μ M LY294002.

4.2.3 Effect of MAPK inhibition on endothelial sprouting

The MAPK pathway is a well characterised downstream effector cascade of Flk-1 signalling in adult endothelial cells (Doanes et al., 1999; Glikli et al., 2001) and in chapter 3 I demonstrated that ERK activation also occurred upon VEGF stimulation of embryoid body-derived cells (section 3.3.2). Additionally, MEK-1 over-expression increases VEGF mRNA levels in fibroblasts (Milanini et al., 1998). *In vitro* experiments using endothelial cell lines and *in vivo* models of tumourigenesis have demonstrated a strong link between inhibition of the MAPK pathway and decreased tumour vascularisation and the MEK-1^{-/-} mouse dies at E10.5 due to defects in placental vascularisation (reviewed in Depeille et al., 2007), indicating involvement of MEK-1 in developmental vasculogenesis. In order to investigate the involvement of MEK signalling in vasculogenesis and angiogenesis in a developmental model, embryoid bodies within the endothelial sprouting assay were incubated in the presence of the pharmacological MEK1/2 inhibitor U0126 in one or both phases of the assay. Previous work in chapter 3 demonstrated that VEGF-stimulated activation of ERK1/2 was completely inhibited by pre-incubation with 10µM U0126. However, embryoid bodies failed to form upon incubation with 10µM U0126 (this finding is discussed in more depth in section 4.3) therefore 5µM U0126 was used in further studies.

Figure 4-8 illustrates that treatment of developing or sprouting embryoid bodies with the MEK1/2 inhibitor U0126 had no significant effect on the ability of embryoid bodies in the endothelial assay to form sprouts. Inhibition of this pathway therefore does not appear to have any effect on vasculogenesis or angiogenesis in embryoid bodies. This tallies with evidence from the MEK-1 knockout mouse in which embryonic vascularisation was unaffected, despite defects in placental vascularisation (Giroux et al., 1999), and the MEK-2 knockout mouse which was viable (Belanger et al., 2003), though the lack of a double MEK-1/MEK-2 knock-out makes it difficult to postulate whether redundancy between the two kinases masks any potential embryonic defect in vasculogenesis.

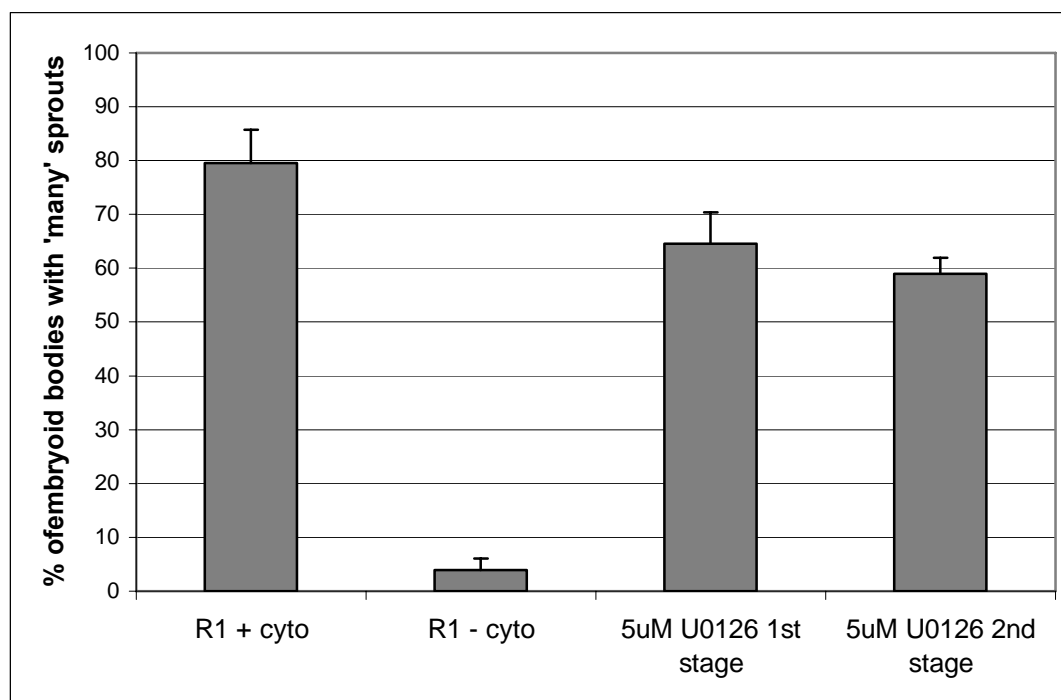


Figure 4-8 Comparison of sprout formation in the presence and absence of the MEK1/2 inhibitor U0126. R1 primary embryoid bodies were cultured for 10 days prior to being placed in a collagen matrix for 2 days to promote sprouting, indicative of angiogenesis. In both stages embryoid bodies were cultured in the presence or absence of a cytokine cocktail and within cytokines present, in the presence and absence of the MEK1/2 inhibitor U0126. The percentage of embryoid bodies sprouting was quantified using the parameters set out in section 4.2. Results shown represent pooled data from 3 independent experiments. The % of embryoid bodies from each culture condition which exhibited 'many' sprouts, indicative of sprouting angiogenesis, are illustrated. Error bars represent s.e.m. ANOVA demonstrated no significant differences between the cytokine treated embryoid bodies in the DMSO control and those cultured in the presence of U0126.

4.3 Effect of MAPK inhibition on embryoid body development and Flk-1 expression

As part of the investigation into the importance of signalling down-stream of Flk-1 in embryonic vasculogenesis (and angiogenesis) the MEK-1/2 inhibitor U0126 was utilised in the endothelial sprouting assay (see section 4.2.3). However, attempts to differentiate R1 embryonic stem cells into embryoid bodies in the presence of 10 μ M U0126 failed. No or very few embryoid bodies formed and those which did form were much smaller than their wild type equivalents.

In order to investigate whether MEK-1/2 inhibition had any effect on the proportion of cells within the embryoid bodies expressing Flk-1 or the level of Flk-1 expression, flow cytometry was used to investigate Flk-1 expression in embryoid bodies treated with the pharmacological inhibitors U0126 and PD98059. U0126 is a non-competitive inhibitor of MEK-1 and 2 kinase activity whereas PD098059 inhibits activation of MEK-1. The IC₅₀ for both inhibitors appears to differ depending on the agonist used. However, PD098059 cannot readily be used at concentrations greater than 50 μ M due to low aqueous solubility (Alessi et al., 1995). Both inhibitors are reportedly selective at the concentrations utilised.

4.3.1 Effect of MAPK inhibition on embryoid body development

Previous work by Burdon *et. al.*, using PD98059, an inhibitor of MEK-1 activation, demonstrated increased self-renewal of ES cells and an inhibition of embryoid body growth, particularly at higher concentrations (100 μ M). This was thought to be due to a defect in differentiation rather than proliferation as the number of cells expressing Oct-4 (a marker of ES cell self-renewal) within the treated embryoid bodies was not only proportionally higher but the absolute number of self-renewing cells increased as the embryoid bodies developed (Burdon et al., 1999). The study used the hanging drop aggregation method of embryoid body formation (see Figure 2-1). ES cells were differentiated in hanging drops in the absence of LIF for 2 days prior to incubation in suspension in the presence or absence of PD98059 thus any early effects of MEK-1 inhibition may have been missed. Additionally, though only noted at higher

concentrations of inhibitor, visual inspection of photographs of embryoid bodies appears to show a decreased size even in the presence of only 25 μ M PD098059.

R1 ES cells were differentiated into embryoid bodies in the presence or absence of 5 μ M U0126 or 50 μ M PD098059 for 4 days before the numbers of embryoid bodies formed were counted. The diameter of 50-80 embryoid bodies cultured under each condition were measured under a light microscope, using an eye piece graticule, at 200X magnification. A counting grid was used to randomise the embryoid bodies measured.

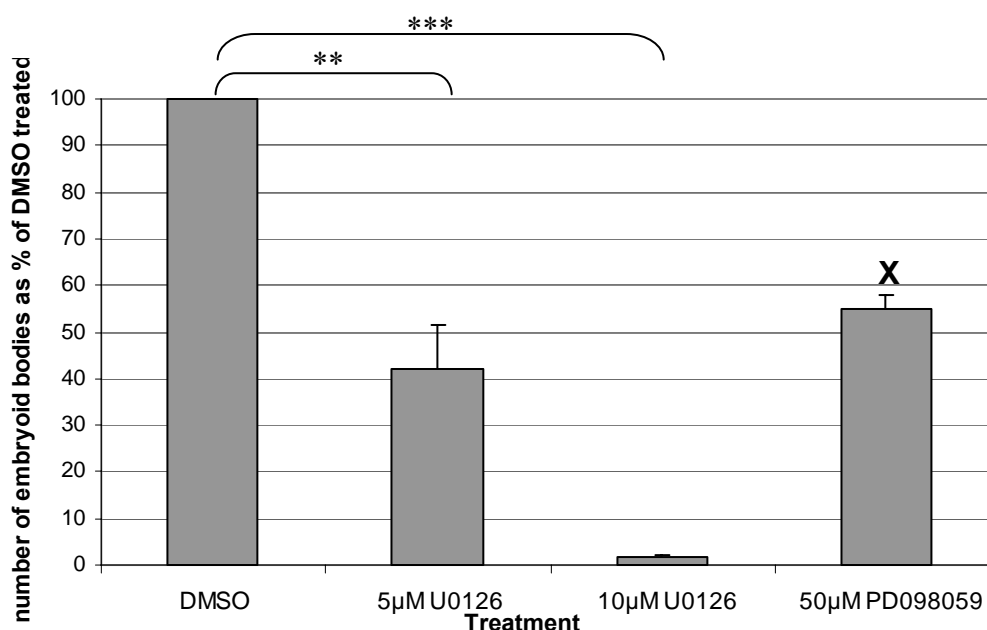


Figure 4-9 Comparison of number embryoid bodies formed in the presence of DMSO control or the MAPK inhibitors U0126 and PD98059. R1 ES cells were differentiated at low density (to form between 100 and 1000 embryoid bodies in the DMSO control) to form embryoid bodies using a methylcellulose-based differentiation system (see section 2.2.1) in the presence of DMSO, 5 or 10 μ M U0126 or 50 μ M PD098059. After 4 days the number of embryoid bodies formed under each culture condition in duplicate 2cm Petri dishes were counted. The chart represents these figures as a percentage of the DMSO control using pooled data from individual experiments as follows; DMSO control and 5 μ M U0126 n=6, 10 μ M U0126 n=3, 50 μ M PD098059 n= 2. Error bars represent s.e.m with the exception of that annotated X which represents the range. **p \leq 0.05, *** p \leq 0.01 in the paired students t-test performed on raw data.

Figure 4-9 illustrates that ES cells differentiated in the presence of the MEK-1/2 inhibitor U0126 formed significantly less embryoid bodies than those cultured in the presence of a DMSO control (see Figure 4-10). Very few embryoid bodies formed in the presence of 10 μ M U0126 and those which did form were much smaller than the DMSO control EBs. ES cells differentiated in the presence of 50 μ M PD098059 showed a decrease in embryoid body formation similar to that seen in the presence of 5 μ M U0126.

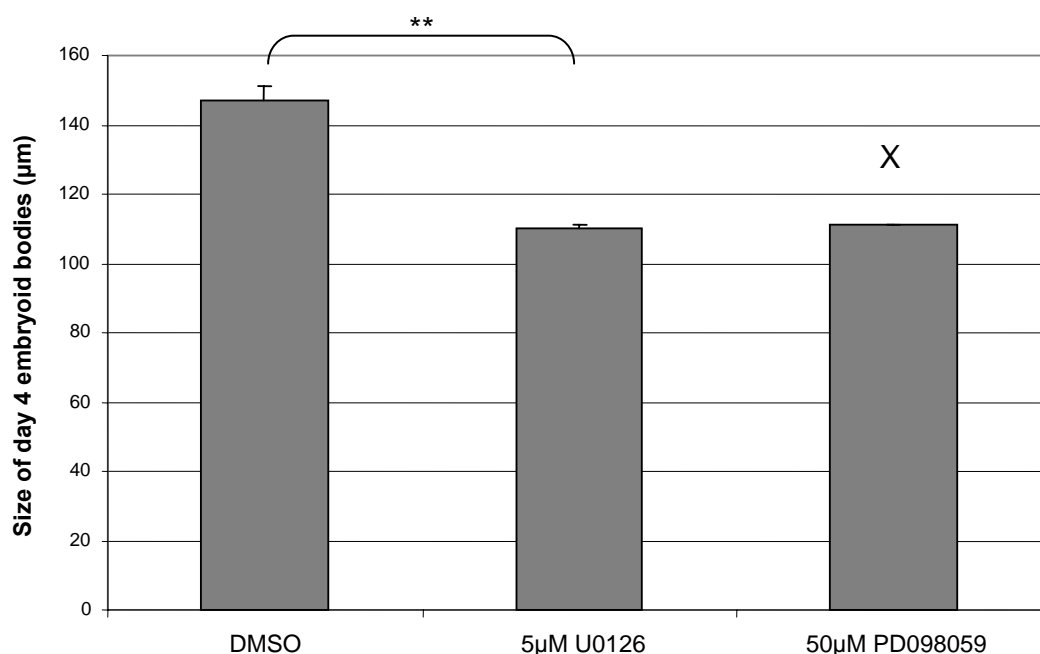


Figure 4-10 Comparison of embryoid body size between EBs formed in the presence of DMSO control and those formed in the presence of MAPK inhibitors U0126 or PD98059. R1 ES cells were differentiated at low density (to form between 100 and 1000 embryoid bodies in the DMSO control) to form embryoid bodies using a methylcellulose-based differentiation system (see section 2.2.1) in the presence of DMSO, 5 μ M U0126 or 50 μ M PD098059. After 4 days 50-80 embryoid bodies formed under each culture condition were measured under a x200 objective using an eye piece graticule. The chart represents the mean sizes of embryoid bodies under 3 separate conditions. DMSO control and 5 μ M U0126 data is representative of pooled data from 3 individual experiments. 50 μ M PD098059 data (annotated X) is representative of only one individual experiment. Error bars represent s.e.m. ** $p \leq 0.05$ in the paired students t-test.

Figure 4-10 illustrates that differentiation of embryoid bodies in the presence of 5 μ M U0126 caused a significant decrease in embryoid body size. Embryoid bodies

differentiated in 10 μ M U0126 also appeared significantly smaller than the DMSO control. However, due to their scarcity there was not a large enough population for a fair comparison to be made. The one set of embryoid bodies differentiated in 50 μ M PD098059 which were measured showed a decrease in size which correlated well with that seen in embryoid bodies differentiated in 5 μ M U0126.

4.3.2 Effect of MAPK inhibition on Flk-1 expression.

Inhibition of the MAPK pathway with the pharmacological inhibitors U0126 and PD098059 decreased both the ability of ES cells to differentiate to form embryoid bodies and the size of subsequent embryoid bodies. In order to investigate whether this decrease in size effects the proportion of Flk-1 expressing cells within the embryoid bodies, ES cells were differentiated as EBs for between 4 and 6 days in the presence or absence of the inhibitors prior to flow cytometric analysis of Flk-1 surface expression.

Inhibition of MAPK signalling with either U0126 or PD098059 caused a slight apparent decrease in Flk-1 expression on days 4 and 5 post-embryoid body formation compared to the DMSO control illustrated in Figure 4-11A. Analysis of variance performed on geometric mean fluorescence intensity data showed these differences as non-significant (Figure 4-11B). This suggests that inhibition of the MAPK pathway does not affect the ability of embryoid bodies to differentiate to form BL-CFC or later endothelial cells. Further analysis of additional mesodermal, haemopoietic and endothelial markers would be required to confirm this hypothesis (see section 4.5.3 for further discussion).

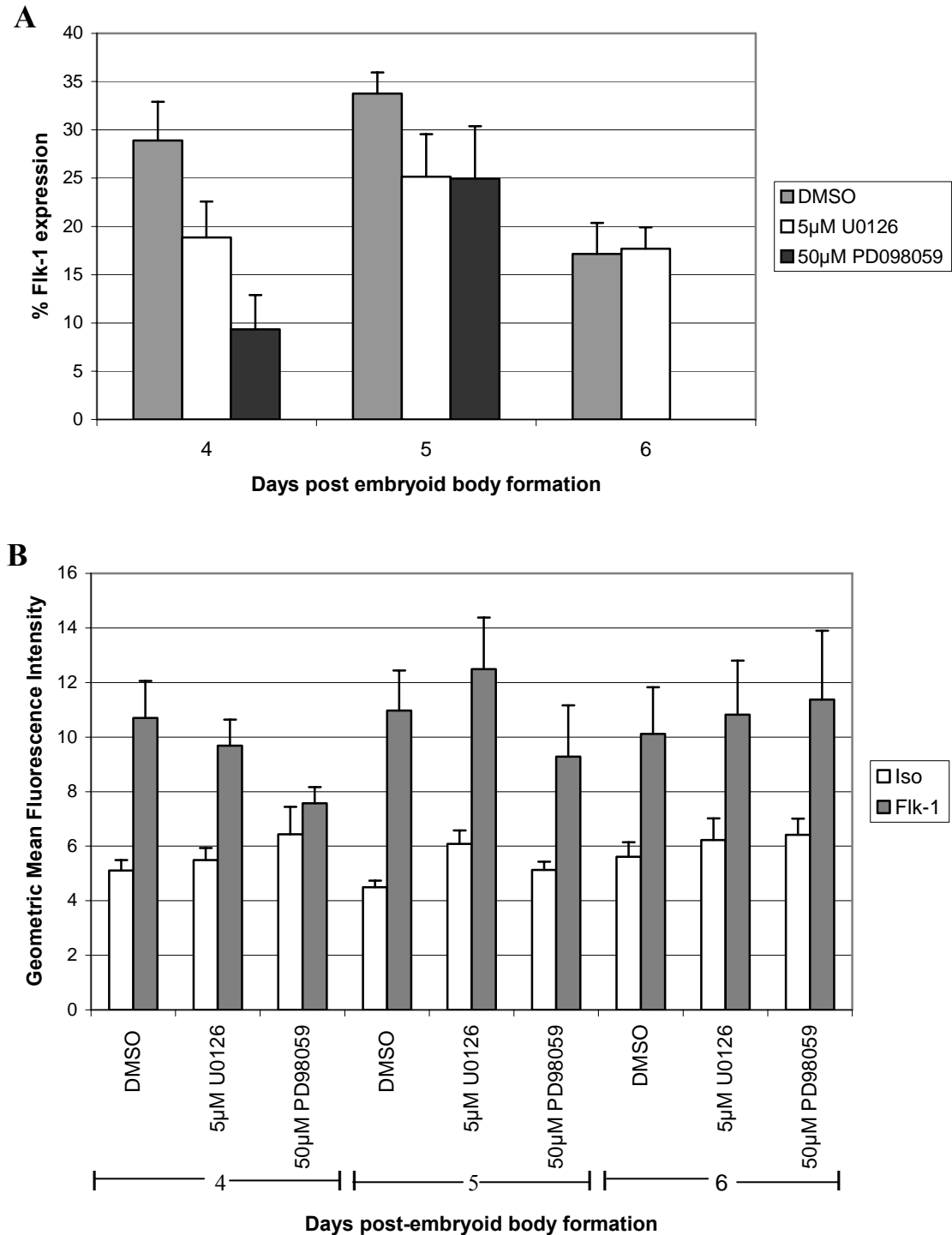


Figure 4-11 Time-course of Flk-1 expression in cells from dissociated embryoid bodies treated with DMSO (control), 5µM U0126 or 50µM PD098059. Cells from dissociated embryoid bodies differentiated for between 4 and 6 days, were stained with an anti-Flk-1 antibody or an equal concentration of isotype-matched control and a secondary FITC-labelled antibody prior to analysis on a Becton Dickinson FACS Canto flow cytometer. The percentage of cell expressing Flk-1 and the geometric Mean Fluorescence Intensity were calculated as described in section 3.2.1. Percentage Flk-1 expression was calculated using a 5% marker gate. The results shown represent pooled data from ≥ 3 individual experiments.

A) Comparison of % Flk-1 expression in cells from dissociated primary embryoid bodies differentiated for between 4 and 6 days in the presence of either DMSO (control), 5 μ M U0126 or 50 μ M PD098059. **B)** Comparison of the geometric mean fluorescent intensities between isotype control and anti-flk-1 labelled cells from dissociated R1 embryoid bodies differentiated for between 4 and 6 days in the presence of either DMSO (control), 5 μ M U0126 or 50 μ M PD098059. Error bars represent s.e.m. Analysis of variance (ANOVA) showed no significant differences geometric mean fluorescence intensities between anti-Flk-1-labelled cells from treated and un-treated dissociated embryoid bodies.

4.4 Importance of Flk-1 in the differentiation of ES cells to form beating cardiomyocytes

In addition to its importance in endothelial and haemopoietic development, recent work has implicated a role for Flk-1 in cardiogenesis (Iida et al., 2005). Lineage tracing experiments identified a Flk-1 positive intermediate population which appears to develop as cells exit the primitive streak and begin to migrate towards the cardiac crescent (Ema et al., 2006). Other studies, using embryoid bodies as a model of cardiogenesis (Boheler et al., 2002; Sachinidis et al., 2002), have demonstrated an increase in expression of cardiac markers in response to VEGF. Work by the Keller group first identified that the cardiogenic mesoderm population was derived from a population which was *Brachyury* positive but Flk-1 negative at E3.25 the point of BL-CFC/haemangioblast formation (Kouskoff et al., 2005). Their work has subsequently demonstrated that at E4.25 a new Flk-1 positive population is generated which was identified to contain the majority of precursors with cardiac potential within the differentiation model and which had the potential to form cardiac, endothelial and visceral smooth muscle lineages (Kattman et al., 2006).

Despite a strong association between Flk-1 expression and cardiac differentiation, a functional requirement for Flk-1 in cardiogenesis had not been proven. To investigate a possible functional requirement for Flk-1 in *in vitro* cardiac development a hanging drop-based differentiation protocol, which promotes formation of beating cardiomyocytes, was used to compare the timing and efficiency of cardiomyocyte differentiation between R1 parental and Flk-1^{-/-} ES cells.

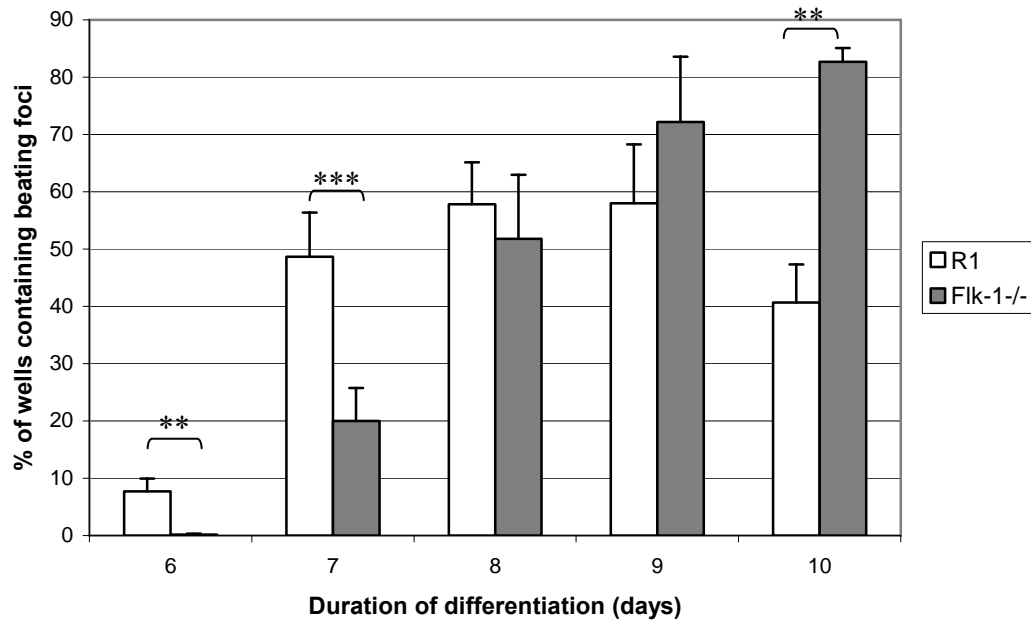


Figure 4-12. Comparison of beating cardiomyocyte formation between R1 (wild-type) and Flk-1^{-/-} ES cells in an embryoid body-based model of cardiac differentiation. R1 and Flk-1^{-/-} ES cells were differentiated in hanging drops for 2 days and in suspension for a further 2 days prior to plating of individual embryoid bodies into gelatine coated wells of a 96-well plate. 96 wells were scored for the appearance of beating foci daily from 6 to 10 days after the initiation of the assay. The above chart represents pooled data from 5 individual experiments. Error bars represent s.e.m. ** $p \leq 0.05$, *** $p \leq 0.01$ in the paired students t-test.

Figure 4-12 above demonstrates an apparent delay in differentiation of cardiomyocytes in Flk-1^{-/-} ES cells compared with the R1 parentals. Though the timing of differentiation within the assay differed by up to a day between experiments (hence the relatively high error bars), the relationship was constant. The initiation of beating areas within Flk-1^{-/-} embryoid bodies begun later than that of the wild type and the percentage of embryoid bodies which differentiated to form beating foci were significantly lower on days 6 and 7 of the assay. From day 8 of the assay there was no difference in the appearance of beating foci between the 2 cell lines and by day 10 when the foci of differentiated R1 cells had begun to stop beating the percentage of Flk-1^{-/-} embryoid bodies which had differentiated to form beating foci was significantly higher than that found in R1 wells at the same time point.

However, there was no significant difference in the highest percentage of wells exhibiting beating foci between differentiated R1 and Flk-1^{-/-} ES cells. By day 10 growth of both cell lines had outstripped the confines of the 96 well plate making it impossible to continue the experiment. This data suggests a potential role but not a requirement for Flk-1 in cardiomyocyte differentiation.

4.5 Discussion

Functional assays were used to explore the role of Flk-1 and Flk-1-mediated signalling in *in vitro* assays probing endothelial and cardiac development. Additionally, pharmacological inhibitors of the MAPK pathway were used to investigate its role in embryoid body development.

4.5.1 Summary

- In the endothelial sprouting assay, used to investigate endothelial specification and subsequent angiogenic expansion, significant decreases in the percentage of embryoid bodies undergoing sprouting were observed in both ES cell lacking Flk-1 or when wild-type R1 ES cells treated with the pharmacological inhibitor VEGFR2 kinase inhibitor 2.
- Withdrawal of extrinsic VEGF from the sprouting endothelial assay caused a significant decrease in the number of embryoid bodies which underwent endothelial sprouting.
- Inhibition of the PI3K pathway with the inhibitor LY294002 had no effect on endothelial sprouting.
- Inhibition of the MAPK pathway with the MEK-1/2 inhibitor U0126 or the MEK-1 inhibitor PD098059 had no significant effect of endothelial sprouting.
- Inhibition of the MAPK pathway with MEK1/2 inhibitor U0126 or the MEK-1 inhibitor PD098059 decreased the efficiency of embryoid body formation

by approximately 50%. The embryoid bodies which did form were decreased in diameter by approximately 25%.

- Flk-1 expression in embryoid bodies cultured in the presence of the MAPK inhibitors for between 4 and 6 days was not significantly altered when compared with DMSO (control)-treated embryoid bodies.
- Flk-1^{-/-} ES cells underwent a delay in differentiation to form foci of beating cardiomyocytes when compared with R1 parental ES cells. When the first beating foci were observed in differentiated R1 cells no beating areas were visible in differentiated Flk-1^{-/-} cells. A day later there were still significantly fewer beating foci in differentiated Flk-1^{-/-} embryoid bodies compared to R1 parental embryoid bodies.
- The potential of Flk-1^{-/-} ES cells to differentiate to form beating cardiomyocytes, though temporally delayed, was equivalent to that of R1 parental ES cells.

4.5.2 The role of Flk-1 and its downstream signalling in endothelial development

The requirement of Flk-1 for both embryonic vasculogenesis and adult angiogenesis is well characterised (Shalaby et al., 1995; Zachary, 2001). In chapter 3 some of the signalling pathways activated downstream of Flk-1 in an embryoid body model of development were elucidated. In order to characterise the importance of these signalling molecules in endothelial development an embryoid body-based ‘sprouting angiogenesis’ assay was used to assess the effects of inhibiting these pathways on vasculogenesis and subsequent angiogenesis within embryoid bodies. Prior to dissection of the roles of these pathways the importance of Flk-1 signalling as a whole was characterised through the use of both genetic and pharmacological approaches.

4.5.2.1 Importance of VEGF and Flk-1 in sprouting from primary embryoid bodies

The percentage of Flk-1^{-/-} embryoid bodies with ‘many’ sprouts, indicative of sprouting angiogenesis was significantly lower than the percentage of sprouting R1 parental embryoid bodies. Feraud *et al.*, reported that ~50% of sprouting projections from wild type embryoid bodies cultured in the presence of angiogenic cytokines were endothelial but that none of those found on embryoid bodies cultured in the absence of angiogenic cytokines were endothelial in nature (Feraud et al., 2001). As there was no significant difference between the percentage of sprouting R1 embryoid bodies in the absence of angiogenic cytokines and the percentage of Flk-1^{-/-} embryoid bodies which sprouted this may suggest that sprouting of the Flk-1^{-/-} embryoid bodies was non-endothelial in nature. Whole mount EB staining for endothelial markers such as Pecam1, Flk-1 and VE-Cadherin, as performed by Vittet *et al.*, would enable clarification of the endothelial nature of the sprouts. Immunocytochemical analysis of the sprouts using antibodies against endothelial markers such as Pecam1 could clarify this (Vittet et al., 1996).

Culture of R1 embryoid bodies in the presence of 5µM of the Flk-1 inhibitor VEGFR2 kinase inhibitor-2 (KI-2) during either the endothelial specification or angiogenic expansion stages of the assay caused a decrease in endothelial sprouting, similar to that seen in Flk-1^{-/-} embryoid bodies. This suggests, as would be expected, that Flk-1 is required both for the formation of angiogenic precursors and for vasculogenesis and sprouting angiogenesis. Culture of R1 embryoid bodies in the presence of 5µM of the Flk-1 inhibitor VEGFR2 kinase inhibitor-1 (KI-1) in the first phase of the assay also caused a significant decrease in the percentage of embryoid bodies with ‘many’ sprouts though not to the extent of that seen using either KI-2 or the Flk-1^{-/-} ES cell line. There are a number of potential explanations for these observations. The decreased effect of KI-1 could be due either to incomplete inhibition of Flk-1 or instability of the inhibitor in long-term culture. However, as optimisation of an α-phospho-Flk-1 antibody for immunoblotting had been unsuccessful at this point both dose-optimisation and stability studies for the Flk-1 inhibitors were difficult to perform. As endothelial cells first emerge in the centre of embryoid bodies (Risau et al., 1988; Vittet et al., 1996; Wang et al., 1992) it is also

possible that penetration of either one of the inhibitors into the embryoid bodies was insufficient to inhibit Flk-1 in differentiating endothelial cells. Furthermore, we cannot rule out the possibility that each inhibitor also inhibits additional kinases, which may also be involved in endothelial development. For example, at higher doses, KI-2 is known to inhibit PDGF β R, which recruits pericytes/vascular smooth muscle cells to the endothelium of nascent blood vessels in response to PDGF secreted by endothelial cells. Although the PDGF β R knockout mouse is perinatally lethal, it exhibits no defects in endothelial or major blood vessel formation (Hellstrom et al., 1999; Soriano, 1994) suggesting that inhibition of PDGF β R would not necessarily affect the outcome of the endothelial sprouting assay. To further assess the effects of KI-1 and KI-2, characterisation of their ability to inhibit Flk-1 signalling in long-term culture could be carried out in the D4T endothelial cell line using immunoblotting with an anti-phospho-Flk-1 antibody. Dose response studies with each of the inhibitors in the endothelial sprouting assay would also help to elucidate whether the differences seen in the ability of KI-1 and KI-2 to inhibit sprouting were due to differing potency's of the inhibitors in the assay or off-target effects.

Feraud *et al.*, also demonstrated an 80% decrease in endothelial sprouting in the absence of extrinsic VEGF (Feraud et al., 2001) and Ng et al., demonstrated a requirement for VEGF in vessel formation but not endothelial specification (Ng et al., 2004). The results reported in this thesis demonstrate a significant decrease in sprouting upon removal of VEGF from the angiogenic cytokine cocktail but this decrease was not as great as that seen upon absence or inhibition of Flk-1. This is probably due to VEGF secretion from cells within the embryoid bodies (demonstrated by detection of VEGF mRNA in day 11 embryoid bodies) instigating an autocrine loop of VEGF-Flk-1 signalling.

The characterisation of the reduction in sprouting in the sprouting endothelial assay afforded by use of the Flk-1^{-/-} ES cell line and the Flk-1 inhibitor VEGFR2 kinase inhibitor-2 provided a base-line to allow the assay to be used to assess the importance of various signalling pathways downstream of Flk-1 in endothelial development.

4.5.2.2 Effect of PI3K and MAPK inhibition on endothelial sprouting from primary embryoid bodies

Neither PI3K nor MAPK inhibition had any effect on the extent of endothelial sprouting from R1 embryoid bodies. Previous work using LY294002 in embryoid bodies has demonstrated its stability for at least 5 days (Bone and Welham, 2007) by which point any effect upon endothelial specification would have been apparent. As the PI3K pathway was not demonstrably activated down-stream of Flk-1 upon VEGF-stimulation it is perhaps not surprising that inhibition of PI3K signalling with 5 μ M or 10 μ M LY294002 did not affect vasculogenesis or sprouting angiogenesis at this early developmental stage. PI3K signalling has been widely implicated in adult angiogenesis and is a key anti-angiogenic target being explored in cancer chemotherapy (Hennessy et al., 2005). One possible mechanism for the role of PI3Ks is through upregulation of VEGF expression (Jiang et al., 2000). This is supported by evidence from a microarray investigating the effect of inhibition of PI3K with LY294002 in self-renewing ES cells, in which LY294002 treatment caused significant down-regulation of VEGFa and c transcripts (M.J Welham, *pers. comm.*). As VEGF was added exogenously in the assay the effects of decreased VEGF expression would be masked. As removal of exogenous VEGF does not fully prevent sprouting within the assay (see section 4.5.2.1), addition of 5 μ M LY294002 to the assay in the absence of VEGF could be used to further probe the role of this pathway in endothelial development, as would molecular analysis of VEGF expression within the assay in the presence and absence of LY294002 using semi-quantitative or quantitative RT-PCR.

Inhibition of MAPK signalling with 5 μ M U0126 also had no effect on the percentage of embryoid bodies that underwent sprouting within the assay. This suggests that the role of Flk-1 in endothelial differentiation is not mediated through its activation of MAPK, which correlates well with studies in knockout animals as neither the MEK-1 or MEK-2 knockout animals demonstrate defects in embryonic vascularisation though this may be due to functional redundancy (Belanger et al., 2003; Giroux et al., 1999). However, as use of 10 μ M U0126 severely inhibited embryoid body formation (see section 4.5.3) only 5 μ M U0126 was used for this study, meaning it is possible that the MAPK pathway is required for endothelial development but that

residual MEK-1/2 activity meets this requirement. To further investigate the importance of MAPK signalling down-stream of Flk-1 in endothelial development a less blunt approach utilising site directed mutagenesis of tyrosine residues at amino acid positions 951,1175 or 1214, of Flk-1 in order to prevent Flk-1 mediated activation of the MAPK pathway could be used. Finally, activation of the MAPK pathway (though not necessarily down-stream of Flk-1), has also been implicated in the up-regulation of VEGF expression (Milanini et al., 1998). Again, performing the sprouting embryoid body assay in the absence of exogenous VEGF and investigating the effect of MEK-1/2 inhibition of VEGF expression would help to elucidate a potential VEGF-mediated role for the MAPK pathway in endothelial development.

The finding that neither inhibition of the PI3K or the MAPK pathways had any effect on the ability of embryoid bodies in the endothelial sprouting assay to undergo sprouting was somewhat surprising, given published data on the role of PI3K in adult angiogenesis, and suggests key distinctions between angiogenesis in the adult versus early embryo. What other candidates could be involved in the transduction of Flk-1 signalling in endothelial differentiation? The p38 MAPK pathway has been implicated in endothelial migration in adult angiogenesis and is reportedly activated down-stream of Flk-1 in adult endothelial cells (Yashima et al., 2001). However, in contrast, use of the pharmacological inhibitor of p38 SB202190 enhanced neovascularisation in chick chorioallantoic membrane (Matsumoto et al., 2002) making the potential role of p38 MAPK unclear. Signalling via phospholipase-C is another pathway that could be functionally involved in endothelial cell differentiation as the PLC knockout mouse, is embryonic lethal and like the Flk-1 knockout mouse suffers vascular defects (Liao et al., 2001). It is also possible that the role of Flk-1 in early endothelial development is largely involved in migratory responses and that in the absence of Flk-1 the microenvironment of potential endothelial cells does not support their commitment and expansion. This is partially supported by studies in the Flk-1 knockout in which the role of Flk-1 in the formation of appears to be largely migratory (Hiratsuka et al., 2005a; Schuh et al., 1999; Shalaby et al., 1997; Shalaby et al., 1995). Further studies using ES cells expressing conditionally active Flk-1 and mutants of Flk-1 where key phosphorylation sites have been replaced, could help further characterise the Flk-1 signalling pathways required in endothelial development.

4.5.3 Effect of MAPK inhibition on embryoid body development and Flk-1 expression

During the endothelial sprouting assay it was noted that differentiation of ES cells in 10 μ M U0126 severely inhibited embryoid body formation and 5 μ M U0126 decreased both the efficiency of embryoid body formation and the size of the embryoid bodies which formed. Further study indicated a 50% decrease in efficiency of embryoid body formation and a 25% decrease in the diameter (equivalent to 55% decrease in volume and cellularity if embryoid bodies at day 4 are modelled as spherical) of embryoid bodies which formed in the presence of 5 μ M U0126 or 50 μ M PD098059. The 50% decrease in efficiency of embryoid body formation correlates well with findings from Burdon *et al.*, which demonstrated increased self-renewal of ES cells and inhibition of differentiation in the hanging drop differentiation assay upon inhibition of MEK-1 with PD098059 from day 2 of differentiation (Burdon *et al.*, 1999). The decrease in efficiency of embryoid body formation in the presence of 5 μ M U0126 and the lack of differentiation when cells were cultured in 10 μ M U0126 suggests an absolute requirement for MAPK in the initiation of differentiation. This could be further investigated by creating an ES cell line expressing a tet-inducible dominant negative MEK-1 mutant which would allow investigation of the stage(s) of differentiation at which MAPK inhibits differentiation (Mansour *et al.*, 1994). Burdon *et al.*, attributed the decreased efficiency of embryoid body formation to a purely differentiative defect. However, the reduced size of the embryoid bodies which did form in 5 μ M U0126 suggests that the defect may also be proliferative. Endothelial sprouting assays performed have confirmed the ability of embryoid bodies differentiated in the presence of 5 μ M U0126 to form endothelial sprouts (see section 4.5.2.2) and the lack of a significant difference in Flk-1 surface expression between embryoid bodies cultured in the presence and absence of inhibitors supports the notion that differentiation can proceed as normal under these conditions. This would imply that the role of MAPK signalling is two-fold, promoting both differentiation and proliferation. To further investigate the role of the MAPK signalling pathway in controlling cell proliferation during differentiation, the cellularity of embryoid bodies cultured in the presence or absence of U0126 and PD98059 could be measured. This could substantiate whether the decrease in embryoid body size observed when ES cells differentiated in the presence of the two

inhibitors was caused by a decrease in cell number within the embryoid body. In addition, analysis of mRNA and protein expression of markers of pluripotency (including Oct-4, Nanog and Rex-1), and differentiation towards different lineages (including *Brachyury*, *Scl*, *Pax6*, and *Nodal*), using semi-quantitative or quantitative RT-PCR and immunoblotting, immunocytochemistry and flow cytometry respectively, could elucidate whether inhibition of MEK1/2 altered the kinetics or lineage bias of differentiation which could in itself explain the reduction in embryoid body size.

4.5.4 Importance of Flk-1 in the differentiation of ES cells to form beating cardiomyocytes.

The delay in initiation of the development of beating areas in Flk-1^{-/-} embryoid bodies, when compared with R1 embryoid bodies, indicates that Flk-1 plays a role in promoting, but is not required for, cardiomyocyte differentiation. However, further studies using Flk-1 inhibitors or Flk-1 siRNA, or virally introduced Flk-1shRNA to inhibit or knock down Flk-1, are required to confirm the result is not due to a disparity between the two ES cell lines. The lack of a requirement for Flk-1 in cardiomyocyte differentiation appears at odds with other findings which have demonstrated an increase in cardiac markers upon VEGF application (Boheler et al., 2002; Fujimoto and Yanagisawa, 1983; Sachinidis et al., 2002) and more recently a decrease in expression of the cardiac specific homeobox gene Nkx2.5 upon inhibition of Flk-1 with an α -Flk-1 antibody (Chen et al., 2006). However, Nkx2.5 itself is not required for cardiomyocyte differentiation but for later morphogenesis in cardiac development (Lyons et al., 1995) so its down-regulation upon antibody blocking of Flk-1 is not necessarily concomitant with a decrease in cardiomyocyte differentiation. The requirement for Flk-1 itself may therefore be later in cardiac development. Chen *et al.*, also found decreased cardiac marker expression upon MAPK inhibition (Chen et al., 2006). As Flk-1 activates the MAPK pathway it is possible that the delay in cardiomyocyte differentiation could be caused by a deficiency in MAPK signalling which could be overcome by increased activation of another signalling pathway such as FGFR1 (Mima et al., 1995). Expression analysis of cardiac genes such as Nkx2.5, myosin heavy chain, cardiac troponin I bFGF,

CD166 (ALCAM) and GATA-4 may help identify the mechanism behind the delay in cardiomyocyte differentiation.

4.5.5 Conclusions

An embryoid body-based endothelial sprouting assay was used to investigate the role of Flk-1 and its downstream mediators in a developmental model of vasculogenesis and angiogenesis. Use of Flk-1 null ES cells or the Flk-1 inhibitor VEGFR2 kinase inhibitor-2 caused a significant decrease in the number of embryoid bodies which formed sprouts, reducing the percentage of sprouting embryoid bodies to that seen in R1 embryoid bodies differentiated in the absence of angiogenic cytokines. Previous work has shown that in the absence of angiogenic cytokines no endothelial sprouts can form (Feraud et al., 2001) suggesting that Flk-1 is required for formation of endothelial sprouts. Inhibition of the MAPK pathway with 10 μ M U0126 during embryoid body development prevented embryoid body formation. Use of 5 μ M U0126 or 50 μ M PD98059 also demonstrated a significant decrease in the efficiency of embryoid body formation and the size of those embryoid bodies which formed, suggesting a requirement for the MAPK pathway for differentiation (and possibly proliferation of differentiating cells). The effect of MAPK signalling on endothelial sprouting was investigated using partial inhibition of MAPK signalling with 5 μ M U0126. No reduction in the ability of embryoid bodies to form sprouts, or the extent of sprouting from individual embryoid bodies was seen suggesting that the MAPK pathway is not involved in endothelial differentiation or migration and that activation of the MAPK pathway by Flk-1 is not the mechanism by which endothelial differentiation and migration are potentiated. Although activation of PI3K downstream of Flk-1 is important for adult angiogenesis (Hennessy et al., 2005) inhibition of PI3K with LY294002 had no effect on sprouting from embryoid bodies. Previous studies of Flk-1 signalling in embryoid body-derived cells (chapter 3) found no evidence of PI3K activation by VEGF in which model, supporting a diminished role for PI3K signalling in developmental vessel formation. Finally, the ability of Flk-1^{-/-} ES cells to differentiate to form beating cardiomyocytes with the same efficiency as R1 parental ES cells (all be it with a delay in differentiation) suggests that despite a strong association between Flk-1 expression and cardiac differentiation

(Boheler et al., 2002; Ema et al., 2006; Kattman et al., 2006; Kouskoff et al., 2005; Sachinidis et al., 2002) Flk-1 is involved in but not *required* for *in vitro* cardiomyocyte differentiation.

Chapter 5

Creation and characterisation of Flk-1-Tet-on ES cell lines

5.1 Introduction

Deletion of Flk-1^{-/-} in the mouse results in embryonic lethality by E9.5 due to a lack of formation of both the vasculature and blood (Shalaby et al., 1995). Owing to this embryonic lethality, use of *in vitro* differentiation systems represents an attractive alternative approach for further investigation of the role of Flk-1 in early embryonic development. Previous work, using an embryoid body-based system of differentiation demonstrated activation of the MAPK and PLC signalling pathways upon VEGF stimulation of Flk-1 in embryoid body-derived cells (section 3.3). In chapter 4 of this thesis, functional assays utilising Flk-1^{-/-} ES cells and inhibitors of Flk-1 and the PI3K and MAPK pathways have been used to begin to investigate the role of Flk-1 signalling in endothelial and cardiac development. Flk-1 expression is required for endothelial differentiation (section 4.5.2, Shalaby et al., 1995) and is important but not necessary for the *in vitro* differentiation of cardiomyocytes (see section 4.5.3). Furthermore, Flk-1 expression may be important in haemopoietic differentiation. Haemopoietic precursors express Flk-1 but it is down-regulated upon lineage commitment (Kabrun et al., 1997). Cells of the haemopoietic lineage are able to differentiate in the absence of Flk-1, although with much lower efficiency and their differentiation appears to be very much microenvironment dependent (Hidaka et al., 1999; Schuh et al., 1999). For example, Flk-1^{-/-} ES cells differentiated *in vitro* in OP-9 stromal cell co-culture can produce only mesodermal plaques and no committed haemopoietic colonies, whilst Flk-1^{-/-} ES cells differentiated in an embryoid body-based system are able to differentiate to form all haemopoietic lineages, all be it at lower efficiency (Hidaka et al., 1999).

In order to further investigate the consequences of varying the timing and duration of Flk-1 signalling on haemopoietic, endothelial and cardiomyocyte development Flk-1 expression was re-constituted in Flk-1^{-/-} ES cells using a Tet-regulated system. Site-directed mutagenesis of tyrosines at amino acid positions 951, 1175, 1214 in the cytoplasmic tail of Flk-1 could then be used to investigate the importance of different Flk-1-mediated signalling pathways during the differentiation process.

5.2 Cloning strategy

The aim of the cloning strategy was to produce an ES cell line which *inducibly* expressed Flk-1. To achieve this, the Tet-regulated expression system in which expression of the gene of interest is controlled by addition and subtraction of the antibiotic tetracycline (or the closely related doxycycline), was used (described in 5.2.1). Previous use of this Tet-regulatable system in the laboratory had occasionally led to mosaic expression of the protein of interest (differing levels of induction between ES cells from the same population; Bone and Paling, unpublished observations). It was postulated that this may have been due to chromatin positional effects. Anastassiadis *et al.*, found some improvement in levels and predictability of expression by flanking the *Tet-o* construct with chicken-globin insulators (Anastassiadis *et al.*, 2002). The pInshygro plasmid, containing four such insulators was therefore used to minimise the possibility of mosaic expression of the tet-response plasmid.

5.2.1 The Tetracycline regulated expression system

The tetracycline-regulated expression system allows high level, regulated expression of a gene of interest. It was developed using the *E.coli* tetracycline repressor protein (TetR) which negatively regulates expression of genes within the tetracycline operon by binding to tet-operator sequences (Tet-o) in the absence of tetracycline (Tet). The mammalian expression system, developed by Gossen and Bujard, comprises two plasmids; a regulator plasmid encoding a version of the TetR protein and a response plasmid, in which expression of the gene of interest is regulated by a promoter containing Tet-o sequences (see Figure 5-1). In the original Tet-off expression system the gene of interest was expressed down-stream of the Tet-operator when tetracycline was withdrawn from culture (Gossen and Bujard, 1992). Modification of four amino acids within the tet-repressor created the modified reverse Tet-repressor (rTetR) of the Tet-on expression system in which addition of doxycycline (a Tet derivative) induces expression of the gene of interest (Baron *et al.*, 1995).

The Tet-on expression system is illustrated in Figure 5-1. The host-cell line must be stably transfected first with the regulator plasmid and subsequently the response plasmid encoding the gene of interest. The regulator plasmid (in this study pCAGGs-

irtTA-VP16-puro;(Anastassiadis et al., 2002) constitutively expresses the reverse tetracycline-transactivator (rtTA) fusion protein comprising the DNA binding domain of the Tetracycline repressor (TetR) fused to the C-terminal 127 amino acids of the transcriptional activation domain of virion protein 16 of herpes simplex virus (VP16), under the control of the CAGGs ubiquitously active promoter (chicken β -actin promoter coupled to human CMV (cytomegalovirus) immediate early enhancer with rabbit β -globin gene sequences including a polyadenylation signal and an SV40 (simian virus 40) origin of replication). The response plasmid (pInshygro-TetTK-Flk-1pA) comprises the Tet-response element (TRE, which consists heptamerised tet-operators), and a downstream minimal herpes simplex thymidine kinase (TK) promoter for the gene of interest. In this study the TRE-TK-gene of interest construct is flanked by four chicken globin insulators.

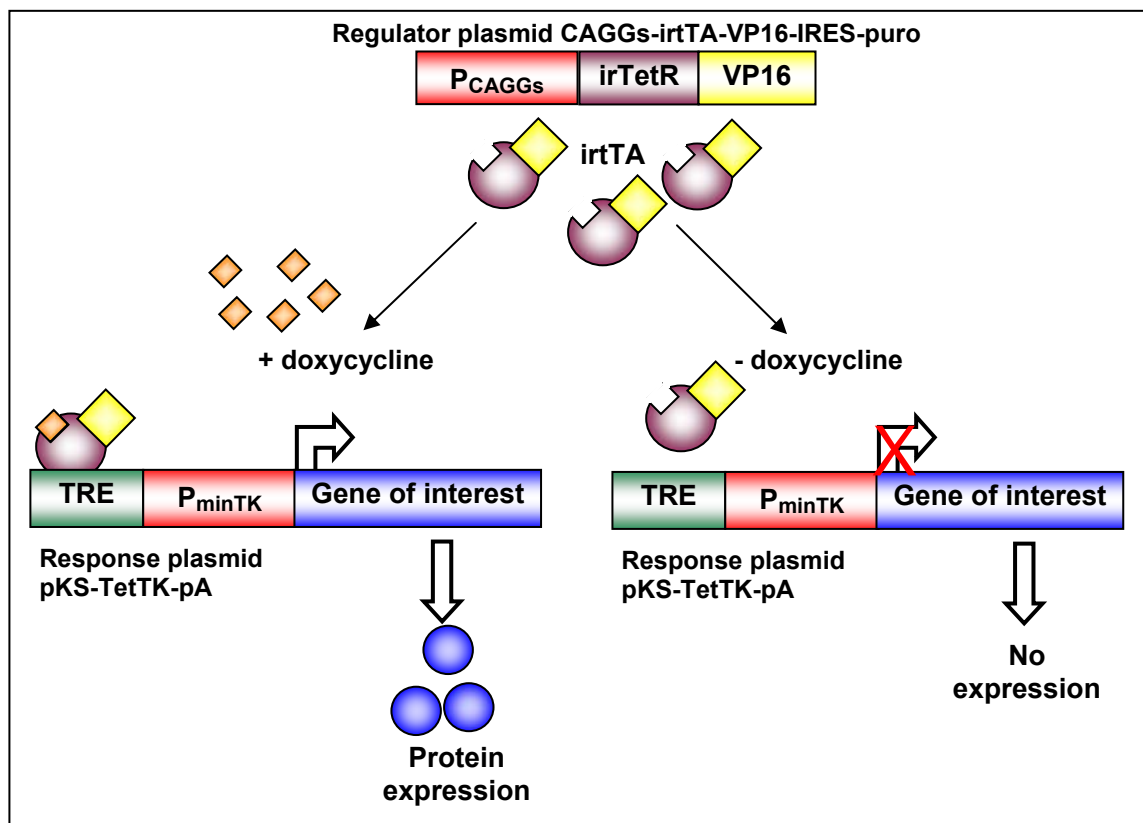


Figure 5-1 Illustration of the Tet-on expression system used in the study. Addition of Doxycycline causes a conformational change in the Tet-transactivator allowing it to bind to the Tet-regulatory element (TRE). This brings the transcriptional activation domain of VP16 into close proximity with the thymidine kinase (TK) promoter allowing transcription of the gene of interest.

The tetracycline-regulated expression system has several advantages over constitutive expression or other inducible systems;

- Tight regulation of gene expression during the generation of stable transfectants with extremely low background or ‘leaky’ expression. This allows study of potentially cytotoxic gene products and in the case of ES cells gene products which may affect self-renewal.
- On/off gene regulation. Unlike the Cre-lox and FRT expression systems in which gene expression is permanently active following homologous recombination the tet-regulated expression system enables rapid up and down-regulation of the target gene allowing temporal expression.
- The prokaryotic regulatory proteins of the tet-regulated expression systems act very specifically on their target sequences with no pleiotropic effects (Harkin et al., 1999).
- Levels of gene expression are comparable or greater than in a constitutive system utilising the same promoter (Yin et al., 1996).
- Both tetracycline or doxycycline at the concentrations required to regulate gene expression in cells are non-toxic and do not induce non-specific effects in contrast to other inducible systems which may require exposure to heat shock, heavy metal ions, steroid hormones, cytokines or radiation (Saez et al., 1997).

5.2.2 Overview of Flk-1 cloning strategy

An overview of the Flk-1 cloning strategy is illustrated in Figure 5-2. The cDNA sequence encoding full length murine Flk-1 was not available commercially and did not appear to have been previously cloned in its entirety. As ES cells differentiated for 3-6 days were known to express Flk-1 (see section 3.2) mRNA containing Flk-1 message was isolated from day 5 primary embryoid bodies. This was then reverse transcribed using random primers and superscript reverse transcriptase. Flk-1 cDNA was amplified from the day 5 primary embryoid body cDNA pool using the proof reading polymerase *Pfu* with sequence specific primers which introduced 5' and 3' XbaI restriction sites. The amplified cDNA was digested with XbaI, gel purified and ligated into XbaI digested pcDNA3.1zeo constitutive expression vector. An EcoRI diagnostic digest was used to establish that Flk-1 was in the correct orientation within the pcDNA3.1zeo plasmid. The construct was then sequenced to confirm the orientation of Flk-1 and verify that the sequence of the cloned Flk-1 was identical to that previously published (GenBank accession number NM_010612) using Sequencher software from Gene Code Corporation. The pcDNA3.1zeo expression vector was used as an intermediary vector in the cloning of Flk-1 into the pKS-TetTK-pA Tet-response vector. This allowed simpler screening and sequencing of cloned Flk-1 as pcDNA3.1zeo is well characterised. The pcDNA3.1zeo-Flk-1 plasmid also allowed constitutive expression of Flk-1 in the Baf/3 pro-B cell line which was used to ensure that Flk-1 could be expressed at the cell surface without the need for an additional signal sequence.

An XbaI digest of the pcDNA3.1zeo-Flk-1 construct released the Flk-1 fragment that was then subcloned into the SpeI-linearised pKS-tettk-pA tetracycline-response plasmid. Once again, diagnostic digests were used to establish the correct orientation of Flk-1 within the plasmid. The tet-tk-Flk-1-pA fragment was excised using blunt end digestion with PvuI and PvuII. The 5kb fragment was gel purified and blunt end ligated into SnaBI linearised pInshygro to create the pInshygro-TetTK-Flk-1-pA plasmid.

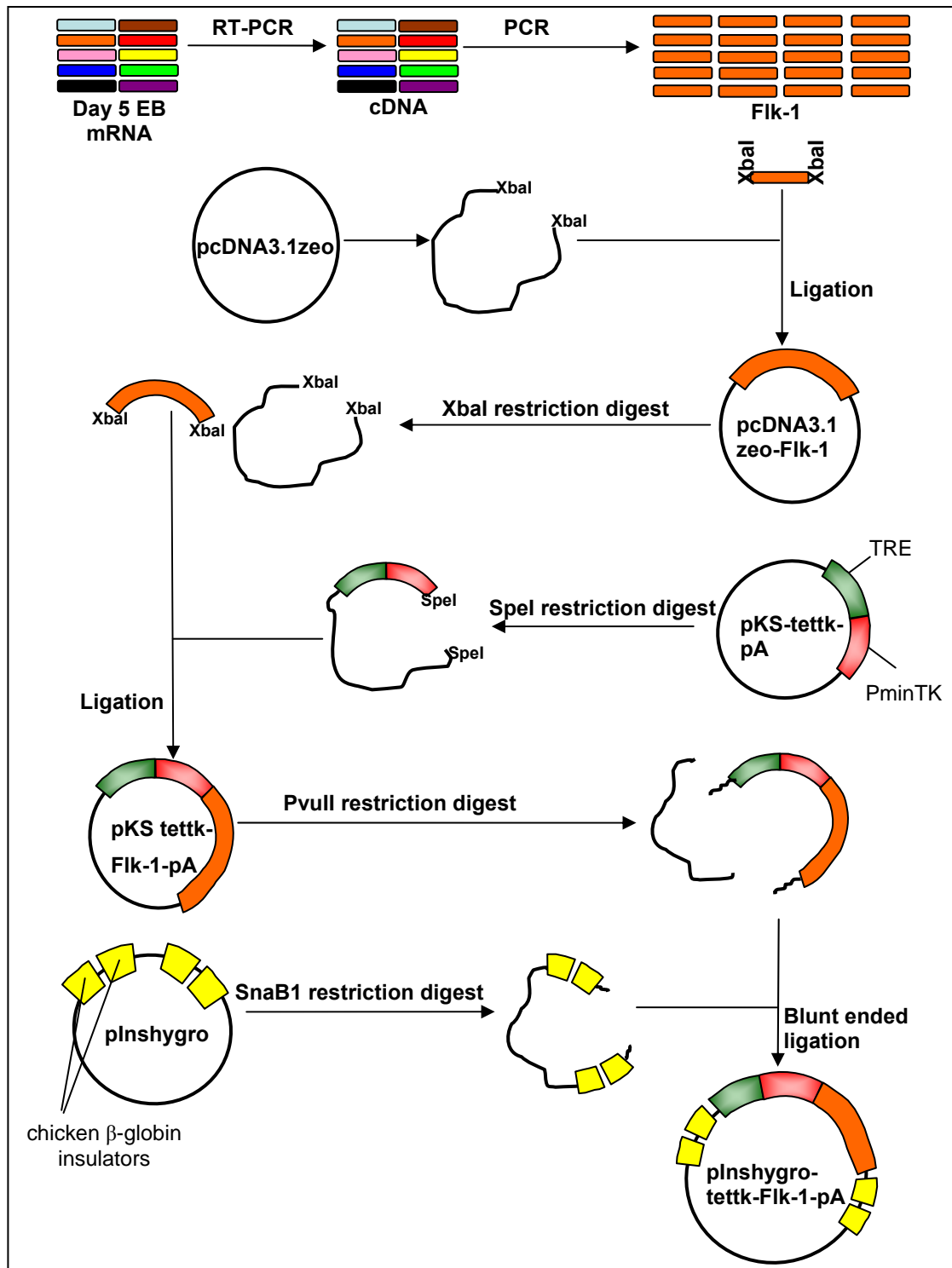


Figure 5-2 Schematic illustrating generation of pInshygro-tettk-Flk-1-pA construct.

Flk-1 cDNA was obtained by RT-PCR from mRNA of day 5 embryoid bodies and amplified with sequence specific primers which added the XbaI restriction site. The Flk-1 fragment was first ligated into pcDNA3.1zeo then sub-cloned into the pKS-tettk-pA plasmid to add the necessary tet-response sequences. Finally the tet-tk-Flk-1-pA fragment was blunt end ligated in the pInshygro plasmid to add chromatin insulators and the hygromycin selection cassette. For plasmid maps see Appendix.

5.2.3 Generation, screening and selection of clones

Initially Flk-1 in the pcDNA3.1zeo construct was transfected into the Baf/3 pro-B cell line by electroporation. Transfected cells were plated at 2×10^5 , 1×10^5 and 0.5×10^5 per well in 96 well plates with Zeocin selection. 37 of 96 in the 0.5×10^5 plate and 58 on both 1 and 2×10^5 plates were Zeocin resistant. 20 clones were picked from the 0.5×10^5 plate and screened by flow cytometry for Flk-1 surface expression. The results are shown in Figure 5-3A. Only clone 9 showed appreciable surface expression of Flk-1 though some expression was also noted in clones 5 and 10. These findings indicate that the Flk-1 cDNA cloned can encode full-length Flk-1 protein. Screening of 3 of the clones apparently not expressing Flk-1 protein using RT-PCR (Figure 5-3B) demonstrated that all 3 expressed Flk-1 mRNA. This result suggests either the clones were expressing a low level of Flk-1 expression or the Baf/3 cells were deficient in an unknown chaperone protein, found in Flk-1-expressing cells, which is required for surface expression.

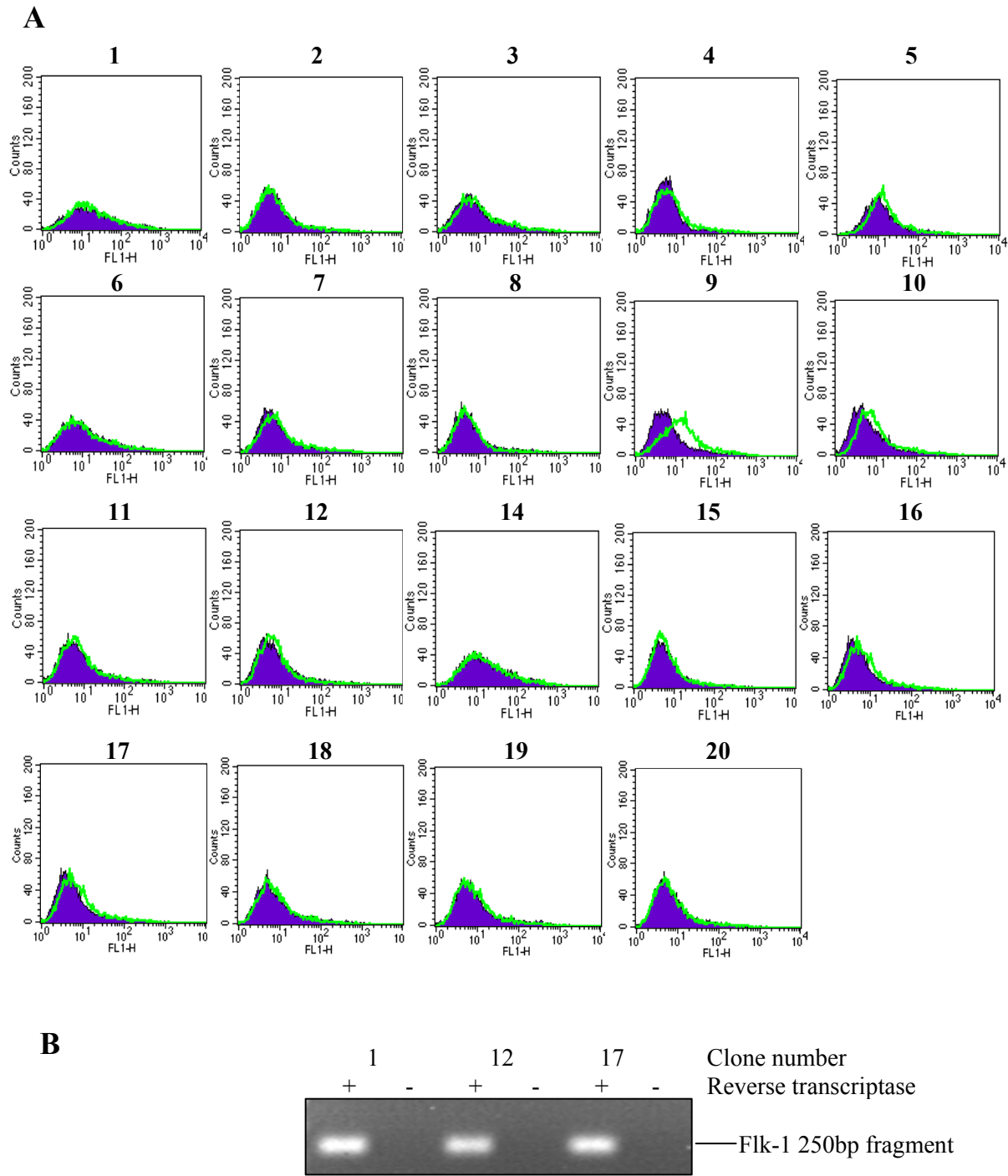


Figure 5-3 Screening Baf/3 clones stably transfected with linearised pcDNA3.1zeo for Flk-1 expression. **A)** Twenty clones were picked and screened for Flk-1 cell surface expression using flow cytometry. Cells were labelled with an anti-Flk-1 antibody or an equal concentration of isotype-matched control and a secondary FITC-labelled antibody. The numbers above the plots indicate the clone identity number. The Y axis represents cell number and the X axis represents fluorescent intensity. The purple trace indicates the isotype control population whilst the green trace indicates the anti-Flk-1 antibody stained population. **B)** mRNA was extracted from clones 1, 12 and 17. RT-PCR was followed by 30 cycles of PCR using primers specific for a 250bp fragment of Flk-1 (see Table 2-9).

To generate Flk-1-Tet-on ES cell lines, Flk-1^{-/-} ES cells underwent two rounds of transfection to create the double stable Tet cell line (see section 2.5.1.2). The first step was to generate Flk-1 null ES cells expressing irtTA. This was achieved by electroporating linearised pCAGGs-irtTA-VP16-IRES-puro plasmid into Flk-1^{-/-} ES cells and selecting positive clones with puromycin. 50 puromycin resistant clones were picked and expanded, 23 were frozen down and 9 were screened (see section 5.2.3.2). In order to screen puromycin resistant clones for the expression of functional irtTA and to compare inducible expression between clones, independent clones were transiently transfected with a pKS-Δp85-tettk-pA Tet-response plasmid which in the presence of doxycycline would express myc-epitope tagged dominant negative p85 (the regulatory subunit of class IA PI3Ks) (see sections 5.2.3.1 and 5.2.3.2). The selected irtTA clones were then stably transfected with the pInshygro-tettk-Flk-1-pA Tet-response construct. Following selection in hygromycin, clones were screened by flow cytometry for doxycycline inducible surface expression of Flk-1 (see section 5.2.3.3).

5.2.3.1 Optimisation of transient transfection of R1 ES cells

Prior to screening of pCAGGs-irtTA-VP16-IRES-puro clones, the conditions for transient transfection in the Flk-1^{-/-} ES cell line were optimised using the pAcΔPHAkt-GFP-N1 GFP expression vector which allowed expression of the transfected plasmid to be quantified using flow cytometry. The electroporation conditions used for stable transfection of the Flk-1^{-/-} ES cell line led to wide-scale cell death and no discernable expression. It was therefore decided to utilise the lipofectamine lipid-based transfection system. The cell number and density, absolute amounts of both DNA and lipofectamine2000 used and the ratio of DNA:lipofectamine are important to maximise expression and minimise cell death. Cell toxicity and efficiency of transfection (assessed by GFP expression) were compared over a range of transfection conditions. The results are illustrated in Figure 5-4.

A

Condition	Cell number	Amount of GFP plasmid	Number of live cells (d3)
50uF, 800V	1×10^6	8 μ g	None (all dead d1)
500uF, 250V	10×10^6 plated 1×10^6	8 μ g	2.5×10^5
500uF, 250V	1×10^6	8 μ g	3.5×10^5
4ul lipofectamine	5×10^5	4 μ g	2.7×10^6
10ul lipofectamine	5×10^5	4 μ g	3.5×10^5
20ul lipofectamine	5×10^5	4 μ g	0.75×10^5
40ul lipofectamine	5×10^5	4 μ g	None (all dead)

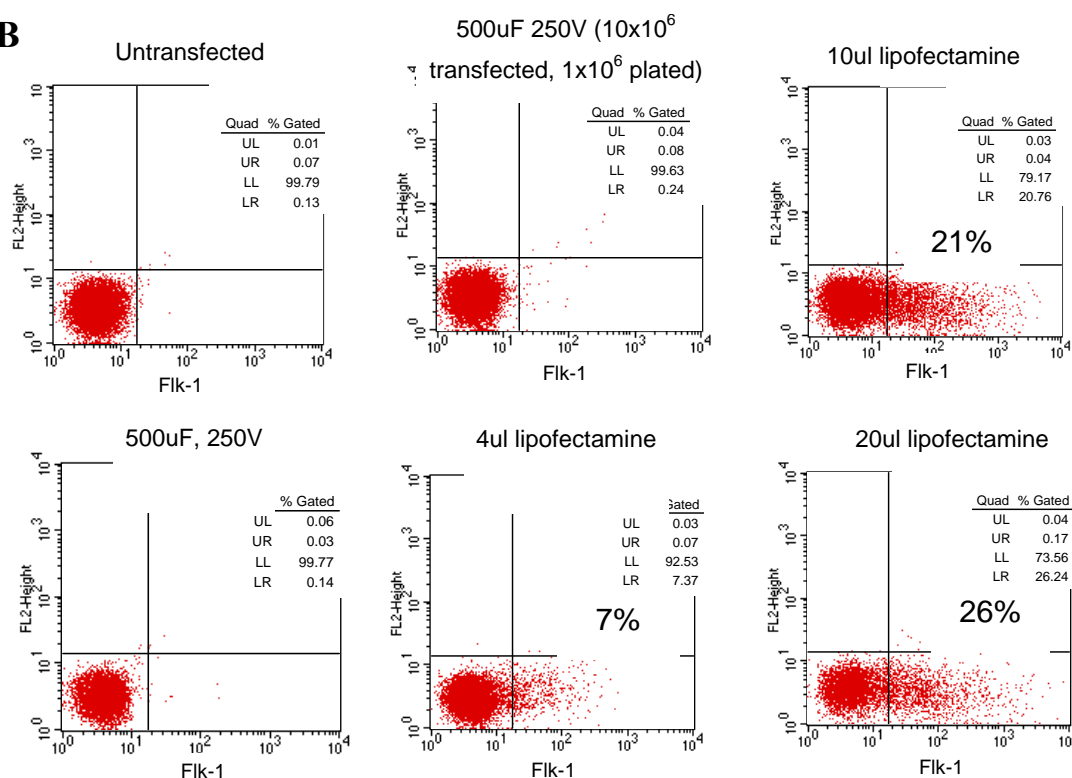
B

Figure 5-4 Optimisation of transient transfection conditions for the R1 Flk-1^{-/-} ES cell line. Flk-1^{-/-} ES cells were transiently transfected with a GFP-expressing plasmid using the conditions described in A. Following transfection the cells were plated onto a 10cm NUNC tissue culture dish. Three days after transfection the number of live cells and percentage GFP expression were measured. **A** illustrates the effect of different transfection conditions on the number of live cells after 3 days. **B** illustrates the percentage of cells expressing GFP and is indicative of transfection efficiency. The experiment was conducted once.

From Figure 5-4B it can be seen that only two of the tested transfection conditions resulted in significant GFP expression, use of 10 or 20 μ l lipofectamine2000 with 4 μ g plasmid DNA and 5 x 10⁵ cells. The transfection efficiency's for both conditions were comparable. However, nearly 5 times more live cells were retrieved from the plate in which 10 μ l lipofectamine2000 had been used therefore this condition was chosen to perform transient transfection of the pKS- Δ p85-tettk-pA Tet-response plasmid expressing myc-tagged Δ p85.

5.2.3.2 Screening *pCAGGs irtTA* clones

To screen the CAGGs *irtTA* reverse-tet-transactivator clones for *irtTA* functionality they were transiently transfected with the pKS- Δ p85-tettk-pA tet-response plasmid. Clones expressing functional *irtTA*, when induced by addition of doxycycline, expressed myc-tagged dominant negative p85 which was detected by immunoblotting with an anti-p85 antibody, shown in Figure 5-5.

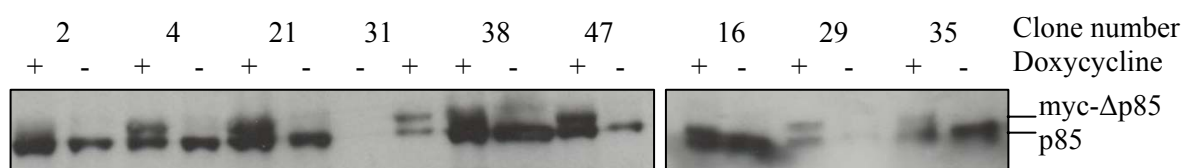


Figure 5-5 Immunoblot screening Flk-1^{-/-} *irtTA* ES cell clones for functional *irtTA*. Flk-1^{-/-} *irtTA* ES cell clones were transiently transfected with the pKS- Δ p85-tettk-pA tet-response plasmid and incubated for an initial 36 hours followed by an additional 24 hours in the presence (+) or absence (-) of 1 μ g/ml doxycycline to induce Δ p85 expression prior to lysis. Whole cell lysates were immunoblotted and probed with an anti-p85 antibody which detected both the wild-type p85 protein and the slightly larger myc-tagged Δ p85.

Figure 5-5 demonstrates that all selected clones inducibly expressed the myc-tagged Δ p85 in the presence but not the absence of doxycycline. The method used for screening makes comparisons of expression levels between clones difficult as they may largely depend on the efficiency of the individual transient transfections. However, clones 29,31 and 47 appear to express Δ p85-myc protein levels equivalent to those of endogenous p85. Assuming transfection efficiency is around 20% (as seen in section 5.2.3.1 using the same conditions), this suggested that in combination with

a stably transfected response plasmid these clones are likely to give good expression. Clone 29 and clone 47 were both tested for their ability to form embryoid bodies. EB formation occurred at approximately the same efficiency as in the R1 parental ES cell line. Clone 29 was selected as the clone to be used for transfection with the pInshygro-TetTK-Flk-1-pA Tet-response plasmid.

5.2.3.3 Screening of Flk-1-tet-ON clones

The Flk-1^{-/-} ES cell line expressing irtTA (clone 29) was stably transfected with the pInshygro-tettk-Flk-1pA construct. Following selection in Hygromycin 40 clones were picked and expanded. Following expansion 22 clones were frozen down and underwent initial screening. The simplest way to screen the clones would have been through lysing the doxycycline-induced cells and immunoblotting with a Flk-1 specific antibody. Unfortunately, optimisation of Flk-1 immunoblotting with 4 separate α -Flk-1 antibodies using the ECL western blotting detection system was unsuccessful, therefore the clones were initially screened using flow cytometry. Each clone was incubated +/- 1 μ g/ml doxycycline for 16 hours to induce expression and screened for doxycycline-induced Flk-1 surface expression by flow cytometry. 13 clones showed no or only slight Flk-1 expression, clone 36 expressed Flk-1 both in the presence and absence of doxycycline and clones 5,10,15,29,32,33,37 and 39 demonstrated varying degrees of doxycycline-induced Flk-1 expression (data not shown). Clones 5, 10, 15, 32 and 33 showed the greatest Flk-1 expression and were therefore subjected to a comparative study.

As demonstrated by Figure 5-6B, all clones reproducibly expressed Flk-1 when induced by addition of doxycycline. Clones 15 and 33 were chosen for use in further characterisation and functional experiments as they expressed the highest dox-induced levels of Flk-1 (Figure 5-6A and B), similar to levels seen in R1 embryoid bodies between days 4 and 5 (see section 3.2.2).

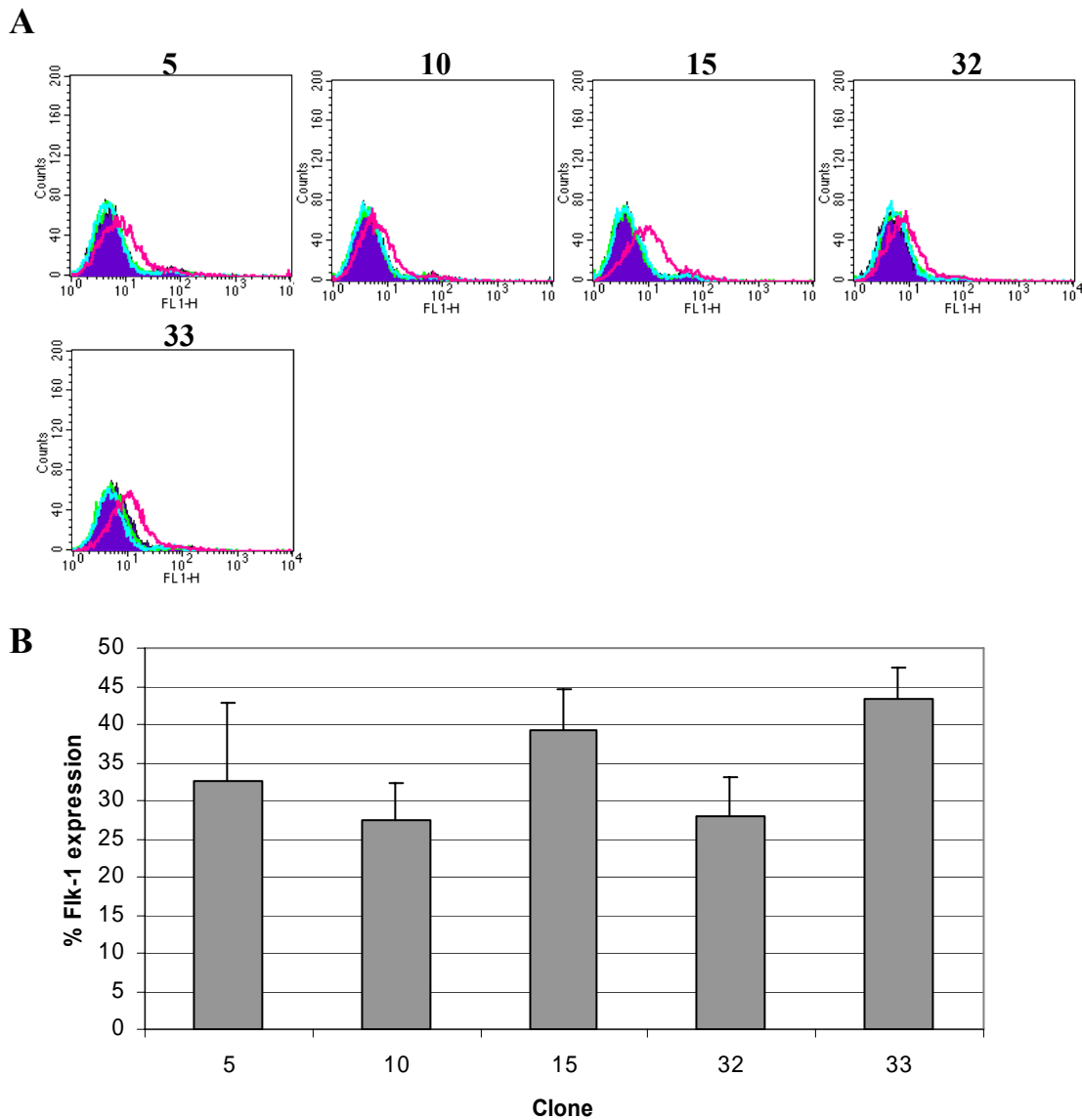


Figure 5-6 Flow cytometric analysis of Flk-1 expression in Flk-1-Tet-on clones. Flk-1-Tet-on ES clones were incubated for 16 hours +/- 1 μ g/ml doxycycline to induce Flk-1 expression. Cells were then labelled with either an anti-Flk-1 antibody or an equal concentration of isotype-matched control, and a secondary FITC-labelled antibody prior to flow cytometric analysis on a Becton Dickinson FACS Vantage flow cytometer. **A)** Histogram plots from one experiment showing comparative Flk-1 expression levels between clones. The numbers above the plots indicate the clone identification number. The Y axis represents cell number and the X axis represents fluorescent intensity. The purple and green traces represent the isotype control and anti-Flk-1 labelled populations respectively in cells incubated in the absence of dox. The blue and pink traces represent the isotype control and anti-Flk-1 antibody labelled cells incubated in the presence of dox. **B)** Percentage Flk-1 expression (calculated using a 5% gate – see section 3.2.1) in Flk-1-Tet-on ES clones in which Flk-1 expression has been induced for 16 hours with 1 μ g/ml dox. Results shown represent pooled data from 3 individual experiments Error bars represent s.e.m.

5.3 Basic characterisation of clones

Before the Flk-1-Tet-on ES clones could be used in any functional assays basic characterisation of the clones were required. These analyses included doxycycline dose optimisation for induction of Flk-1 expression and examination of the timing, duration and functionality of Flk-1 expression following induction both during self-renewing and differentiating states.

5.3.1 Dose-response of Flk-1 expression to varying concentrations of doxycycline

Firstly, induction of Flk-1 expression by varying concentrations of doxycycline was investigated. Flk-1-Tet-on ES cells (clones 15 and 33) were incubated in a range of doxycycline concentrations for 16 hours prior to investigation of Flk-1 expression by flow cytometry and immunoblotting, which was optimised for anti-Flk-1 and anti-p-Flk-1 antibodies using a new ECL-based advanced detection system with significantly enhanced sensitivity compared to systems previously available. The results illustrated by Figure 5-7A and B demonstrate a dose-response of induction of Flk-1 expression to doxycycline. Figure 5-7A indicates that induction is first seen at 10ng/ml dox in clone 15 and 50ng/ml dox in clone 33. Flk-1 protein is visualised in 2 bands of ~210 and 230 kDa, indicating that both partially and fully glycosylated forms of Flk-1 are expressed. Expression in both clones peaks between 100 and 500ng/ml dox and this is born out by Figure5-7B, in which no increase in surface expression is seen between 500ng/ml and 2µg/ml. No Flk-1 expression is seen in clone 15 or clone 33 incubated in the absence of doxycycline confirming tight regulation of expression within the system (also illustrated by anti-Flk-1 immunoblots in Figure 5-8).

1µg/ml doxycycline was used in subsequent experiments as Flk-1 expression is maximal at this concentration and as doxycycline has a 24 hour half-life in culture starting with a higher concentration than is necessary may help to prolong expression. Additionally, no toxic effects have been reported in other cell systems at this concentration.

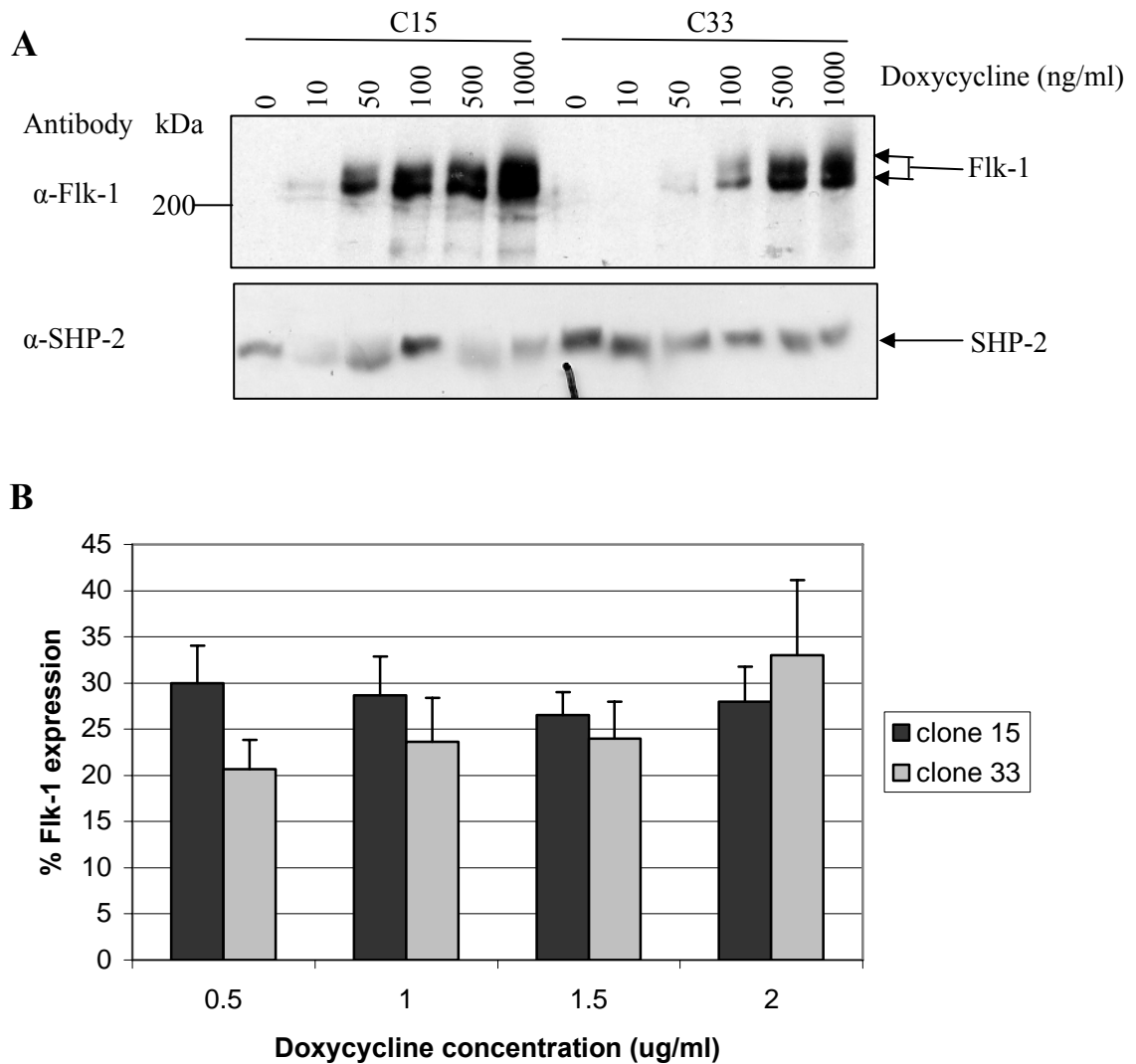


Figure 5-7 Ability of varying concentrations of doxycycline to induce Flk-1 expression in Flk-1-Tet-on clones. Flk-1-Tet-on ES clones were incubated for 16 hours +/- 0-2µg/ml doxycycline to induce Flk-1 expression. **A)** Following doxycycline induction cells were lysed and whole cell lysates immunoblotted with an anti-Flk-1 antibody. Blots were stripped and re-probed with an anti-SHP-2 antibody to check protein loading. **B)** Cells were labelled with either an anti-Flk-1 antibody or an equal concentration of isotype-matched control, and a secondary FITC-labelled antibody prior to flow cytometric analysis on a Becton Dickinson FACS Canto flow cytometer. The percentage of cells expressing Flk-1 was calculated using a 5% marker gate (see section 3.2.1). No Flk-1 expression was seen in the no doxycycline control. Results shown represent pooled data from 2 individual experiments. Error bars represent range.

5.3.2 VEGF-stimulation of Flk-1 in Flk-1-Tet-on ES cells

Upon VEGF stimulation the Flk-1 receptor is auto-phosphorylated at a number of tyrosine residues including Y951 (Zachary, 2001). VEGF-stimulation of day 5 embryoid body-derived cells resulted in receptor phosphorylation that peaked at 5 minutes and decreased to basal by 30 minutes (see section 3.3.1). To check that the Flk-1 protein expressed in the Flk-1-Tet-on ES cell clones was functional, cells were pre-induced with doxycycline, stimulated with 20ng/ml VEGF for between 0 and 30 minutes and immunoblotted with an anti-phospho-Y951 Flk-1 antibody.

Figure 5-8 demonstrates that VEGF stimulation of both clone 15 and clone 33 results in phosphorylation of the receptor over a time-course identical to that seen in VEGF-stimulated day 5 embryoid body-derived cells when Flk-1 expression is pre-induced with 1µg/ml doxycycline. This indicates functionality of Flk-1 expressed in Flk-1-Tet-on ES cells. No Flk-1 expression or phosphorylation is seen in either clone incubated with no dox confirming tight regulation in the system.

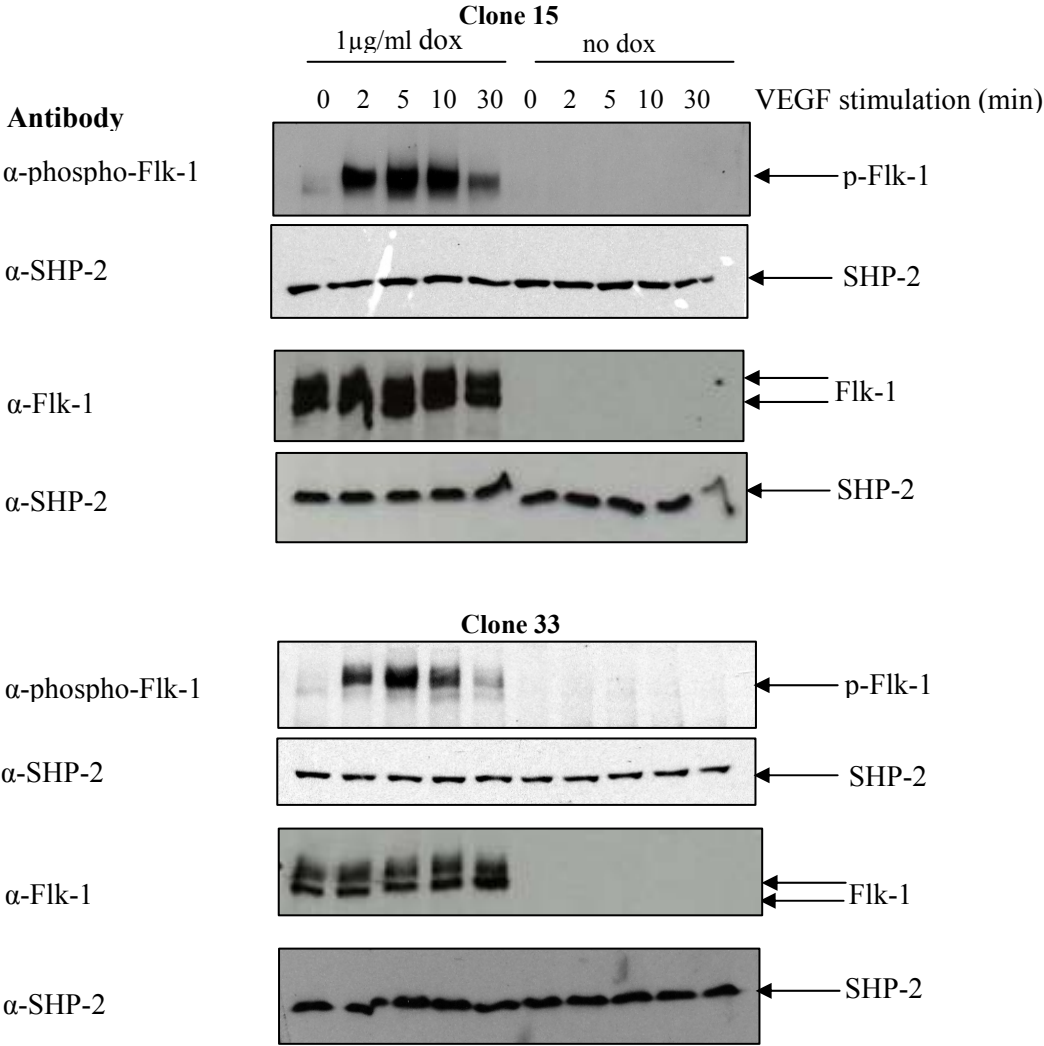


Figure 5-8 VEGF-stimulated Flk-1 phosphorylation in Flk-1-Tet-on ES clones. Immunoblot of whole cell lysates from Flk-1-Tet-on ES clones (15 and 33) pre-incubated in the presence or absence of 1 μ g/ml doxycycline for 24 hours prior to stimulation with 20ng/ml VEGF over a 30 minute time course. Blots were probed with an anti-Flk-1 and anti-phospho-Flk-1 antibodies then stripped and reprobed with the anti-SHP-2 antibody to control for equal protein loading. Blots shown are representative of 3 individual experiments for each clone.

5.3.3 Time-course of Flk-1 expression in Flk-1-Tet-on ES cell clones

The intended use of the Flk-1-tet-ON ES cell line was in differentiation studies therefore the duration of Flk-1 expression following doxycycline induction was characterised in both self-renewing ES cells and models of differentiation. ES cells were incubated in the presence or absence of 1µg/ml doxycycline and Flk-1 surface expression was measured using flow cytometry.

5.3.3.1 Duration of inducible Flk-1 expression in self-renewing ES cells

Diffusion of small molecules such as doxycycline in the embryoid body model of differentiation may be difficult. As self-renewing ES cells grow as a monolayer they provide a more accessible means of characterising inducible Flk-1 expression with which expression during *in vitro* models of differentiation could be compared. Therefore, duration of Flk-1 expression following doxycycline induction was first characterised in Flk-1-Tet-on ES cells cultured in the presence of LIF. To maintain self-renewal ES cells need to be passaged regularly so this experiment was only conducted for 4 days following plating of cells in the presence of doxycycline. Figure 5-9 demonstrates that Flk-1 expression in Flk-1-Tet-on ES cells is maintained for at least 4 days following doxycycline induction.

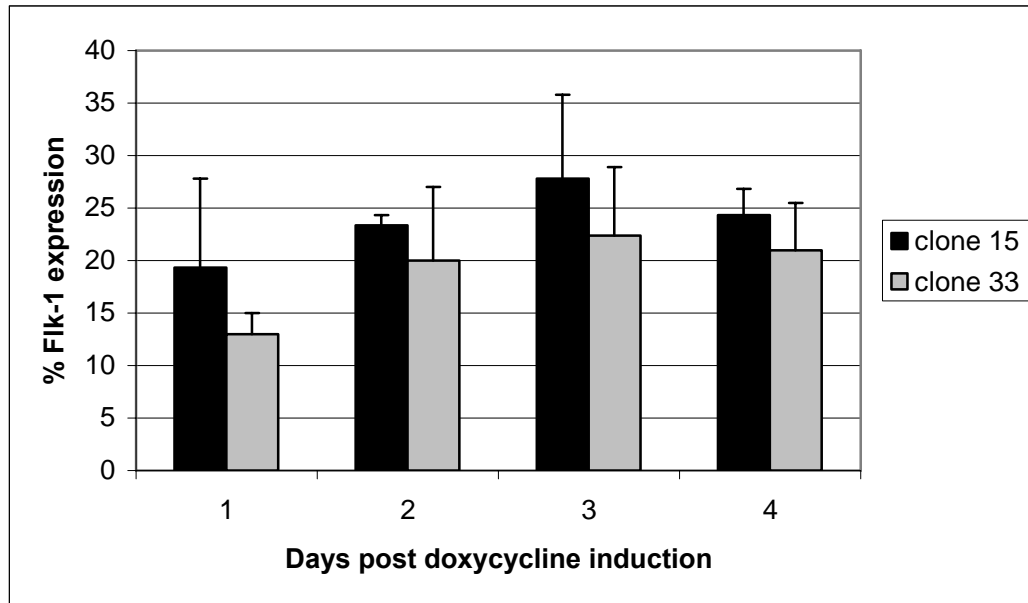


Figure 5-9 Time course of Flk-1 expression in Flk-1Tet-on ES cells following induction with 1 μ g/ml doxycycline. Flk-1-Tet-on ES clones were incubated +/- 1 μ g/ml doxycycline. Doxycycline-induced Flk-1 expression was monitored by flow cytometry for 4 days. Cells were labelled with either an anti-Flk-1 antibody or an equal concentration of isotype-matched control, and a secondary FITC-labelled antibody prior to flow cytometric analysis on a Becton Dickinson FACS Canto flow cytometer. The percentage of cells expressing Flk-1 was calculated using a 5% marker gate (see section 3.2.1). Results shown represent pooled data from 2 individual experiments. Error bars represent range.

5.3.3.2 Duration of inducible-Flk-1 expression during differentiation

Following characterisation of the duration of Flk-1 expression in self-renewing Flk-1-Tet-on ES cells the duration of Flk-1 expression following doxycycline induction needed to be characterised in *in vitro* models of differentiation. The first model in which the duration of Flk-1 expression was characterised was the formation of embryoid bodies in semi-solid medium. As illustrated by Figure 5-10 Flk-1 expression was minimal by day 3 and insignificant by day 4 of differentiation (Flk-1 expression less than 5-10% cannot be measured accurately using flow cytometry as small differences between isotype control and Flk-1 plots could easily be due to chance). It was thought that the decrease in Flk-1 expression may have been due to poor diffusion of doxycycline in the semi-solid medium therefore embryoid bodies were differentiated in liquid suspension. Similar results were seen in Flk-1 expression in these embryoid bodies. Expression greatly declined by day 3 and was

insignificant by day 4. This raised the possibility that diffusion of doxycycline into the embryoid bodies may have at least in part contributed to the decrease in expression. Addition of 1 µg/ml doxycycline to suspension embryoid bodies cultured in its absence at day 2 did not appear to induce any Flk-1 expression in day 4 suspension embryoid bodies nor did addition of additional doxycycline to day 2 suspension embryoid bodies already cultured in 1 µg/ml doxycycline maintain Flk-1 expression to day 4 (Figure 5-10).

Differentiation of Flk-1-Tet-on ES cells in adherent monolayer culture removed any doubt over the access of doxycycline to all cells cultured in the system. This was achieved inducing differentiation of Flk-1-Tet-on ES cells using LIF withdrawal. Flk-1 expression levels in this differentiation system were higher Flk-1 after 3 days but by 4 days no Flk-1 expression was observed suggesting that differentiation itself may have been the trigger of down-regulation of Flk-1 surface expression (Figure 5-10).

It was unclear whether expression of Flk-1 protein had decreased or whether it was possible that differentiation had caused increased internalisation of Flk-1. Gampel *et al.*, reported increased Flk-1-cycling leading to increased Flk-1 expression in human endothelial cells upon VEGF-stimulation (Gampel et al., 2006) and culture of embryoid bodies in the presence of exogenous VEGF in this study increased Flk-1 surface expression in embryoid body-derived cells (section 3-2-4). To investigate whether VEGF increased dox-induced Flk-1 expression in differentiating Flk-1-Tet-on ES cell clones clones 15 and 33 were incubated in the presence and absence of both doxycycline and VEGF. Addition of VEGF at neither day 2 nor 3 had any effect on doxycycline-induced Flk-1 expression (data not shown).

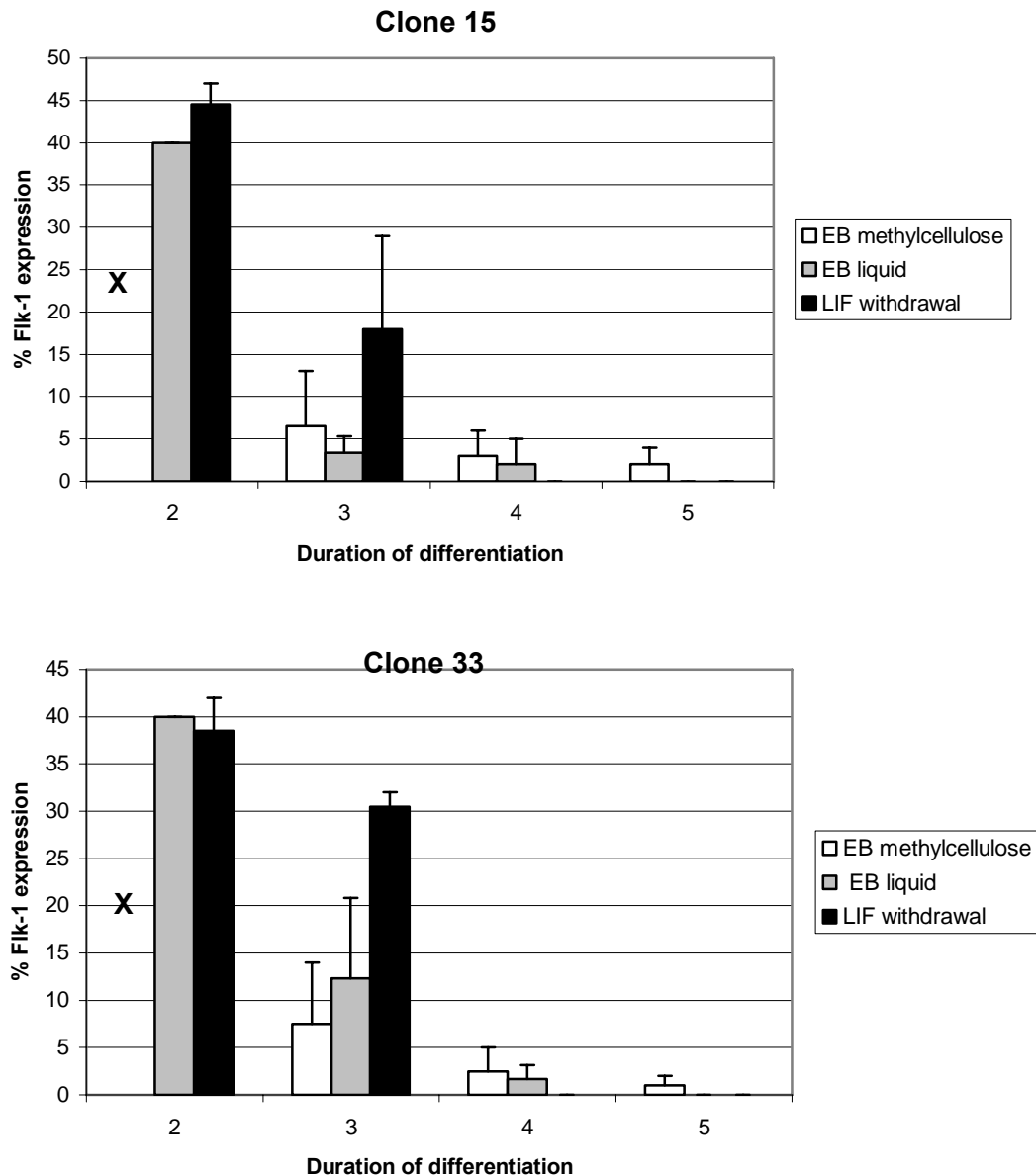


Figure 5-10 Time-course of Flk-1 expression in Flk-1-Tet-on ES cells during differentiation in the presence of inductive doxycycline. Flk-1-Tet-on ES clones were differentiated in the presence of 1µg/ml doxycycline using 3 separate differentiation protocols; embryoid body formation in semi-solid or liquid medium and differentiation of ES cells by removal of LIF. Flk-1 expression was monitored by flow cytometry for 5 days. Cells were labelled with either an anti-Flk-1 antibody or an equal concentration of isotype matched control, and a secondary FITC labelled antibody prior to flow cytometric analysis on a Becton Dickinson FACS Canto flow cytometer. The percentage of cells expressing Flk-1 was calculated using a 5% marker gate (see section 3.2.1). Results shown represent pooled data from 2 individual experiments (X indicates that analysis was not performed). Error bars represent range

Despite a perceived lack of Flk-1 expression in day 4 differentiating EBs whole cell lysates from the same cells did show Flk-1 protein expression (illustrated in Figure 5-11). Unfortunately whole cell lysates from day 4 embryoid bodies were not run in parallel with those from cells from self-renewing ES cells or day 2 embryoid bodies on which Flk-1 surface expression was demonstrably higher. Therefore it is not possible to ascertain whether Flk-1 protein expression was decreased at day 4 or whether the receptor was internalised.

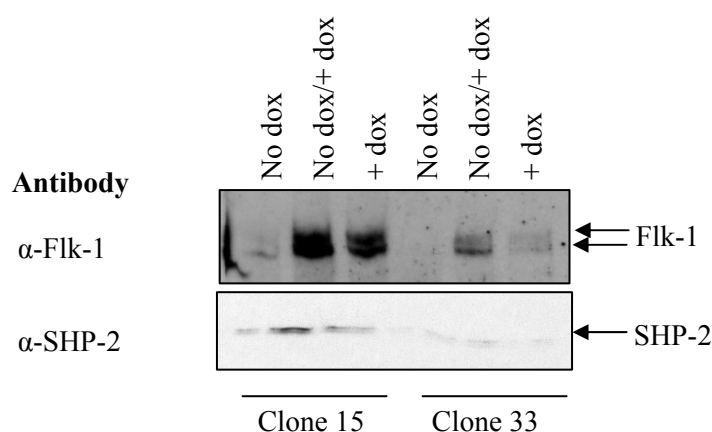


Figure 5-11 Flk-1 expression in day 4 Flk-1tet-ON primary embryoid bodies.

Immunoblots of whole cell lysates from Flk-1-tet-ON ES cells differentiated to form embryoid bodies either in the absence of 1 μ g/ml dox for 4 days (no dox), the presence of dox for 4 days (+dox), the presence of dox for only days 3 and 4 (no dox/+dox) or the presence of dox for 4 days (+dox). Blots were probed with an anti-Flk-1 antibody then stripped and reprobed with an anti-SHP-2 antibody to check for equal loading.

5.4 Functional characterisation of clones

The basic characterisation of the Flk-1-Tet-on ES cells appeared to demonstrate that doxycycline-induced Flk-1 expression may not be maintained during differentiation of ES cells. However, it was decided to investigate whether even a short-term or low level increase in Flk-1 expression could alter cell fate.

5.4.1 Investigation of the effects of temporal changes in Flk-1 expression on cardiomyocyte differentiation of ES cells

Flk-1 is implicated in the differentiation of cardiomyocytes (see section 4-4). In chapter 4 it was demonstrated that Flk-1^{-/-} ES cells exhibited a delay in initiation of beating in the cardiomyocyte assay. As Flk-1 in R1 embryoid bodies is expressed from day 3 onwards the Flk-1-Tet-on ES cell lines were used to investigate whether this delay could be prevented by early induction of Flk-1 expression.

The assay is performed in 3 phases (for more detail see section 2-2). Embryoid bodies are formed in hanging drops and after 2 days transferred into liquid media. Following a further 2 day incubation individual embryoid bodies are plated onto the gelatin-covered wells of a 96-well plate. To investigate whether up-regulation of Flk-1 could rescue the delay in initiation of beating in Flk-1^{-/-} ES cells, 1µg/ml doxycycline was added on day 3 to Flk-1-Tet-on clones. c29irtTA (Flk-1^{-/-} ES cells stably transfected with the pCAGGs-irtTA-VP16-IRES-puro tet-regulator plasmid, the parental cell line of Flk-1-Tet-on ES cells) and R1 ES cells were included as controls.

Both clone 15 and clone 33 of the Flk-1-Tet-on clones as well as c29irtTA cells, incubated in the presence of doxycycline exhibited greatly reduced numbers of beating cardiomyocytes. As the phenotype occurred in the c29irtTA cell line it was obviously not due to dox-induced Flk-1 expression. Further investigation into this phenotype revealed that it occurred only in c29irtTA upon exposure to doxycycline (Figure 5-12C) and did not occur in response to doxycycline treatment in either R1 or Flk-1^{-/-} ES cell lines (Figure 5-12A and B).

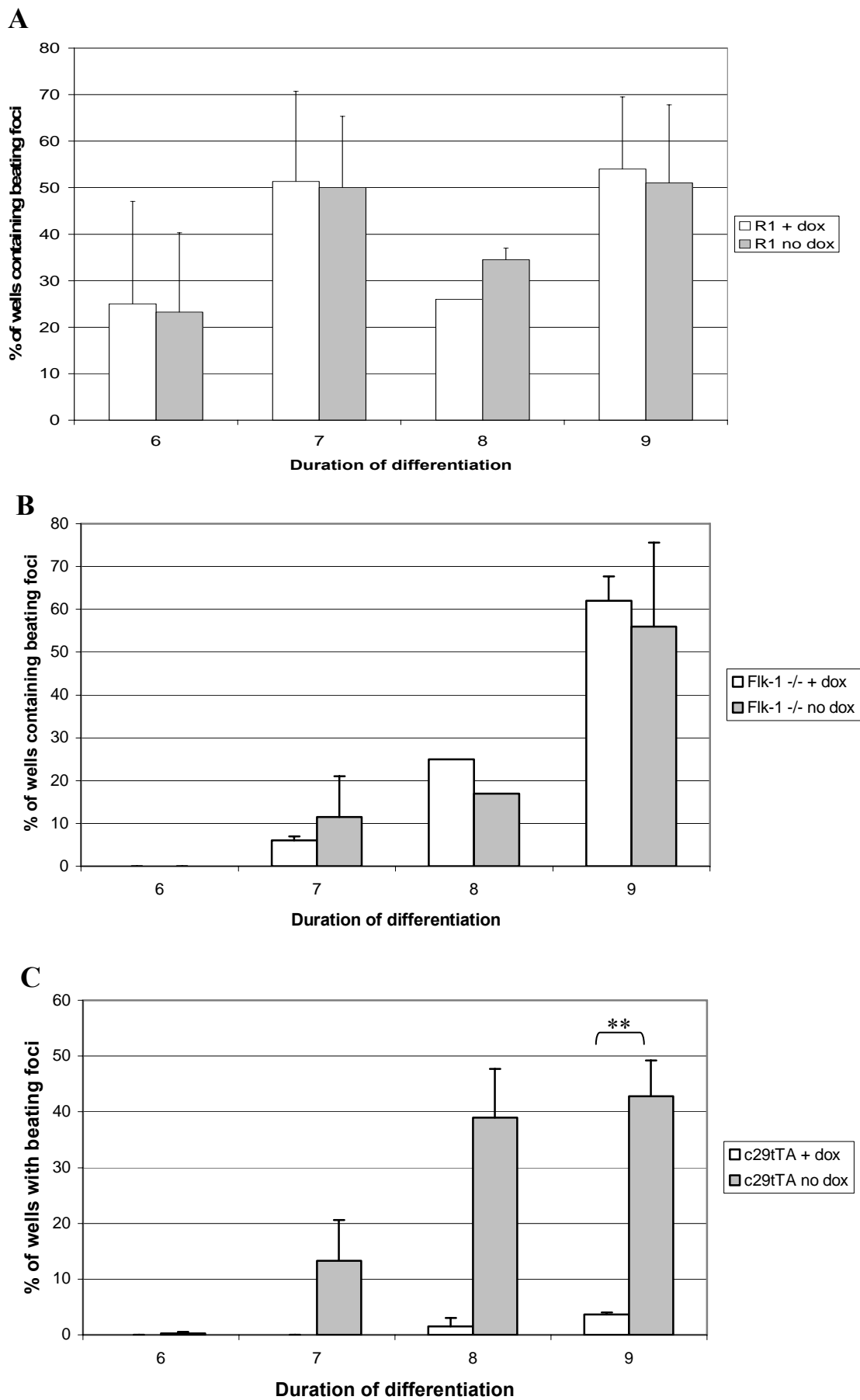


Figure 5-12 continued overleaf

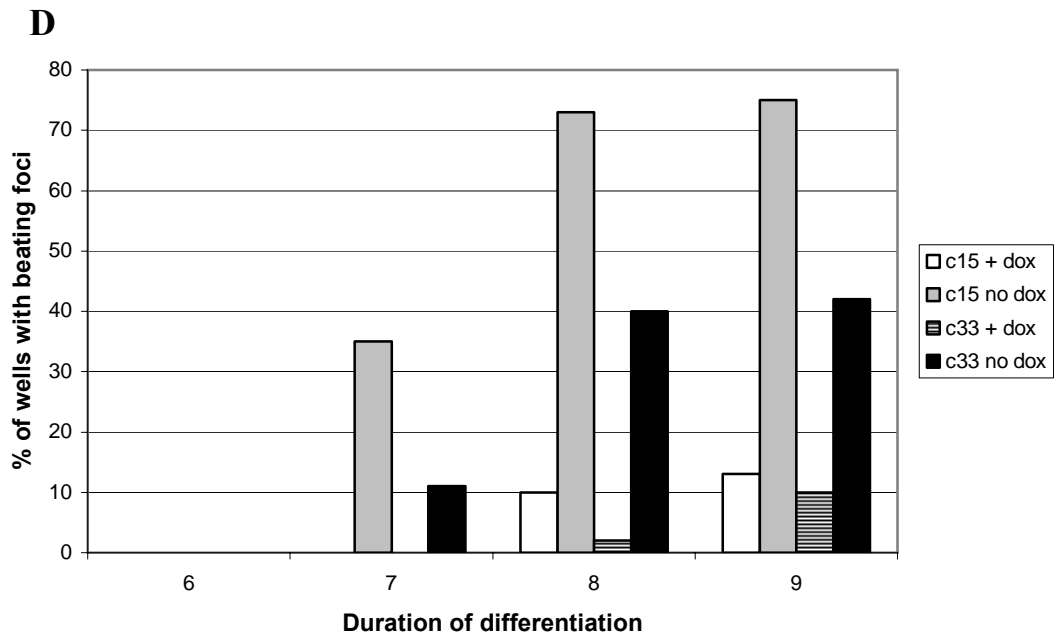


Figure 5-12 Comparison of beating cardiomyocyte development between R1, Flk-1^{-/-} c29irtTA ES cells cultured in an embryoid body-based model of cardiac differentiation in the presence and absence of doxycycline. ES cells were differentiated in hanging drops for 2 days and in suspension for a further 2 days prior to plating of individual embryoid bodies into gelatine coated wells of a 96-well plate. Doxycycline where used, was added at 1µg/ml on day 3. 96 wells were scored for the appearance of beating foci daily from 6 to 9 days after the initiation of the assay. **A)** R1 ES cells **B)** Flk-1^{-/-} ES cells **C)** c29irtTA ES cells **D)** clone 15 and clone 33 of Flk-1-Tet-on ES cell line. Charts A-C represent pooled data from 3 individual experiments. Chart D represents data from one individual experiment. Error bars represent s.e.m. **p≤0.05 in the paired students t-test.

There are several explanations for the effect of doxycycline on formation of beating cardiomyocytes by Flk-1 null ES cells transfected with the irtTA transactivator (C29irtTA). The most likely cause is expression of the VP16 transcriptional activation domain which can interfere with expression of genes lacking VP16 binding sites (Baron et al., 1997). However, toxicity of both rtTA (Bryja et al., 2003) and doxycycline (Moutier et al., 2003) have also been demonstrated in ES cells and murine fetuses respectively. As c29irtTA ES cells could differentiate to form cardiomyocytes in the absence of doxycycline it is unlikely that rtTA toxicity was occurring.

5.4.1.1 DiOC₆ staining

To establish whether the inhibition of cardiomyocyte differentiation was due to toxicity of doxycycline in c29irtTA ES cells 5×10^5 cells were plated onto 10cm tissue culture dishes with 0,1 and 2 μ g/ml doxycycline for 3 days. After 3 days cells were stained with trypan blue and viable cells counted with a haemocytometer, 2.4×10^6 cells viable cells were counted from dishes cultured with 0 or 1 μ g/ml doxycycline and 3.2×10^6 cells were counted from the dish cultured in 2 μ g/ml doxycycline. Very few trypan blue stained cells were observed in any of the cultures. DiOC₆ (3,3'-dihexyloxacarbocyanine iodide) staining was used to investigate whether cells incubated in the presence of doxycycline were undergoing apoptosis. Reduced mitochondrial membrane potential is an early irreversible step in apoptosis that causes a decrease in uptake of the cationic DiOC₆ and a corresponding decrease in FL1 fluorescence measured by flow cytometry. Figure 5-13 illustrates that addition of dox to c29irtTA ES cells did not lead to a decrease in DiOC₆ staining. This combination of results suggests that at the doses used doxycycline was not toxic to the c29irtTA ES cells.

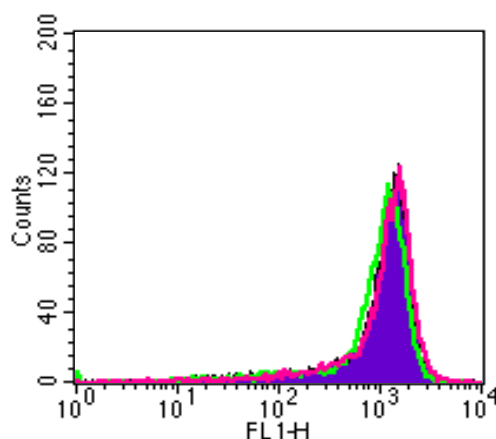


Figure 5-13 DiOC₆ staining of c29rtTA cells incubated for 3 days in the presence of absence of doxycycline. C29rtTA ES cells were incubated for 3 days in 0, 1 or 2 μ g/ml doxycycline prior to DiOC₆ staining. Histogram plots show comparative DiOC₆ staining measured by flow cytometry. The Y axis represents cell number and the X axis represents fluorescent intensity. The purple trace indicates cells incubated in the absence of dox, the green trace indicates cells incubated in 1 μ g/ml dox and the pink trace indicates cells incubated in the presence of 2 μ g/ml dox.

5.4.2 Haemopoietic plaque assay

Mice that lack Flk-1 expression die by E9.5 due to failure of formation of haemopoietic and vascular systems (Shalaby et al., 1995). However, *in vitro* Flk-1^{-/-} embryoid bodies re-plated into a haemopoietic colony assay are able to form all myeloid and erythroid lineages, allbeit at reduced frequency. Conversely, Flk-1^{-/-} ES cells differentiated in OP9 stromal cell co-culture can produce only mesodermal plaques and no committed haemopoietic colonies (Hidaka et al., 1999). This suggests that formation of haemopoietic progenitors is very much microenvironment dependent, a hypothesis which is supported by *in vivo* observations which indicate a requirement for Flk-1 in the migration and expansion of the BL-CFC/haemangioblast rather than its formation (Schuh et al., 1999). Due to the inability of Flk-1^{-/-} ES cells to form haemopoietic colonies in OP9 co-culture this system was used to investigate the requirement for Flk-1 in facilitating haemopoietic differentiation using Flk-1-Tet-on ES cells.

Flk-1-Tet-on ES cells were plated onto semi-confluent OP9 stromal cells to promote the formation of mesodermal plaques. After 2.5 days 1µg/ml doxycycline was added to induce Flk-1 expression in c15 and c33 Flk-1-Tet-on ES cells. It was expected that this would up-regulate Flk-1 expression by day 3, the point at which Flk-1 expression in R1 wild type ES cells is first detected in OP9 co-culture (Paling, *pers. comm*). Day 5 colonies were examined for formation of mesodermal plaques. Representative colonies from R1 wild type ES cells (panels A and B) and c15 Flk-1-Tet-on ES cells (panels C and D) are illustrated in Figure 5-14. It was difficult to positively classify the colonies formed as either mesodermal or undifferentiated possibly because the mesodermal plaques had not had long enough to develop. However, it was noted that R1 colonies were generally larger than colonies formed by any of the Flk-1 null ES cells and that doxycycline treatment (Figure 5-14A and C) did not appear to affect colony appearance in any of the ES cell lines used.

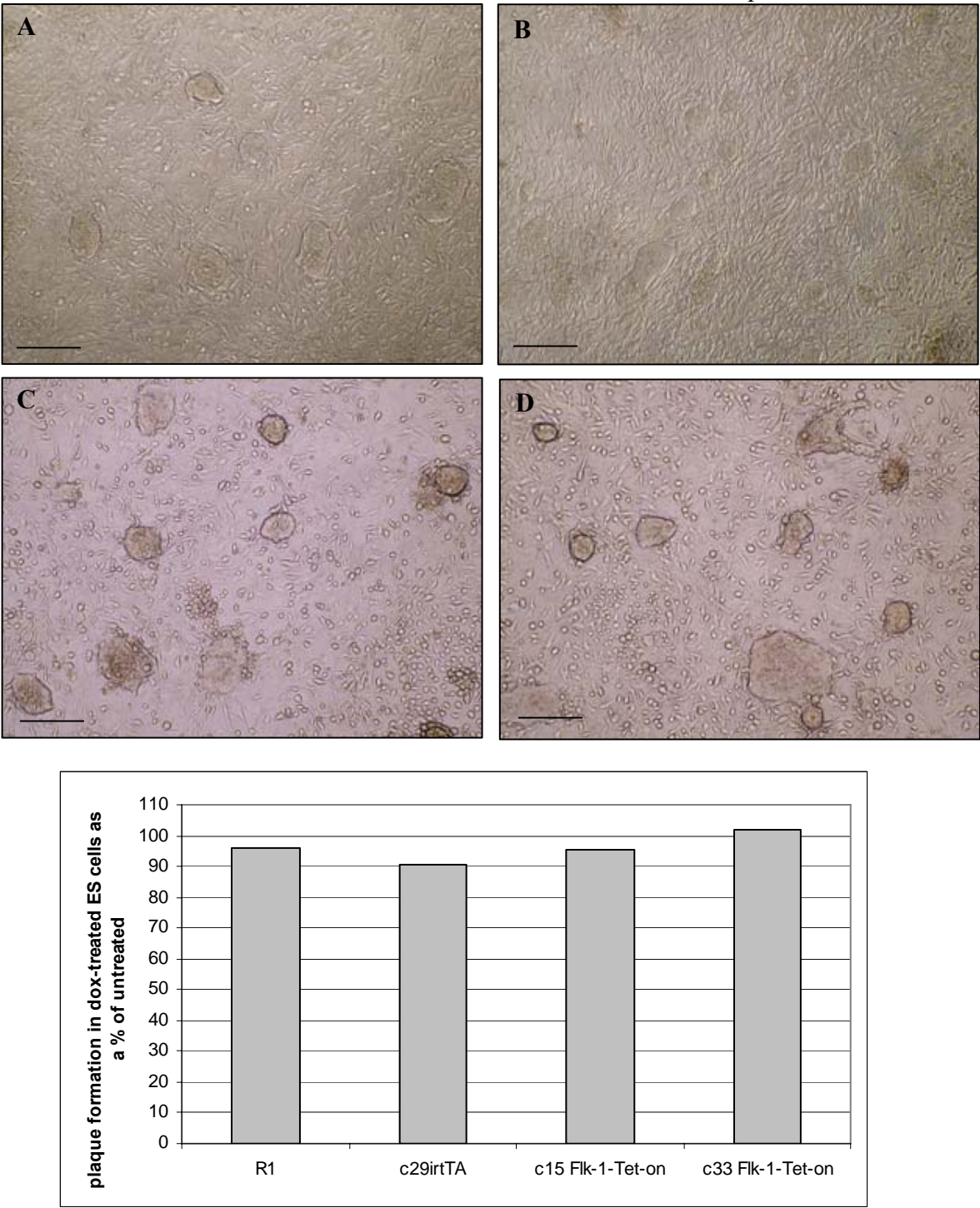


Figure 5-14. Representative plaques from R1 and clone 15 Flk-1-Tet-on ES cells differentiated for 5 days in OP9 co-culture in the presence or absence of doxycycline. R1, c29irtTA and Flk-1-Tet-on ES cells were plated onto semi-confluent OP9 stromal cells in a 6 well plate to promote the formation of mesodermal plaques. After 2.5 days 1µg/ml doxycycline was added to half the wells of each cell line. On day 5 plates were examined for formation of mesodermal plaques. Representative colonies from; **A** and **B**. R1 wild type ES cells, **C** and **D**. clone 15 Flk-1-Tet-on ES cells cultured in the presence (**A** and **C**) and absence (**B** and **D**) of doxycycline. Scale bar 1cm = 250µm. **E**. All colonies (+ and – doxycycline treatment) for each cell line were counted. The number of colonies formed by

doxycycline treated ES cells is expressed as a percentage of those formed by untreated cells. Chart represents pooled data from 3 individual experiments.

Figure 5-14E demonstrates that doxycycline treatment had no effect on the total number of colonies which formed in any of the ES cell lines investigated. Furthermore though total colony numbers for the cell lines used varied there were no observed differences between the cell lines used.

As it was difficult to accurately distinguish between undifferentiated and mesodermal colonies at day 5 and the co-culture protocol called for dissociation and re-plating of colonies at day 5, total colonies were counted (for results see Figure 5-14E above) and RNA was isolated for genetic characterisation (Figure 5-15).

Cells harvested from day 5 colonies were re-plated onto fresh OP9 cells and cultured with fresh doxycycline for a further 5-7 days. RNA was again extracted from day 12 colonies and semi-quantitative RT-PCR was used to investigate expression of mesodermal and haemopoietic markers in R1 wild type, c29rtTA, and Flk-1-tet-ON colonies cultured in the presence and absence of doxycycline. The panel of markers used to investigate haemopoietic differentiation were:

Brachyury – an early mesodermal marker

c-kit – a receptor tyrosine kinase required for haemopoietic differentiation

Scl – a basic helix-loop-helix transcription factor required for primitive haemopoiesis

β -H1-globin – embryonic haemoglobin characteristic of embryonic erythropoiesis.

β -major-globin – adult haemoglobin characteristic of embryonic, fetal and adult erythropoiesis.

These markers were all used by Hidaka *et al.*, to characterise R1 and Flk-1^{-/-} ES cell differentiation in the OP-9 haemopoietic plaque assay (Hidaka et al., 1999). More detail can be found in section 1.3.1.

Figure 5-15 illustrates mesodermal and haemopoietic marker expression in R1 wild type, Flk-1^{-/-} and Flk-1-Tet-on ES cells cultured on an OP9 stromal cell layer to promote multi-lineage haemopoietic differentiation. The primers for Flk-1 itself were

designed against a portion of the gene downstream of the β -galactosidase insert therefore transcript can still be seen in Flk-1^{-/-} ES cells which makes the data difficult to interpret. Future use of Flk-1 primers spanning the β -galactosidase insert site such as those designed by Shalaby *et al.*, would give a clearer picture of doxycycline inducible Flk-1 expression (Shalaby *et al.*, 1997). However, greater levels of transcript are seen on both days in Flk-1-Tet-on clones incubated in the presence of doxycycline suggesting that Flk-1 expression was up-regulated on both day 5 and day 12 of the assay. In addition, immunoblotting of lysates from day 12 clone 15 and c33 Flk-1-Tet-on clones encouragingly indicates some induction of Flk-1 by doxycycline (Figure 5-15B).

Brachyury expression was decreased in Flk-1^{-/-} cultures compared to wild-type on day 5 suggesting that there may be a lower proportion of cells of the mesodermal lineage. This result contrasts with that seen by Hidaka *et al.*, who reported no decrease in *Brachyury* expression on day 5. However, by day 12 *Brachyury* expression in Flk-1 null cells had increased indicating that the colonies are more immature than those formed from wild type R1 ES cells in which *Brachyury* expression was down-regulated.

Expression of the haemopoietic markers however, did follow the pattern previously reported. Flk-1^{-/-} ES cells differentiated in OP9 co-culture expressed little or no *Scl*, β -H1-globin and β -major-globin. Expression of β -H1-globin appeared slightly higher in doxycycline treated c15 and c33 Flk-1-Tet-on cells which may indicate upregulation of haemopoiesis by induction of Flk-1 expression. However, the observed increase is very slight and would have to be further characterised before conclusions could be drawn.

There was no clear pattern in expression of c-kit which was expressed at days 5 and 10 in all ES cell lines and did not appear to be further induced by doxycycline treatment. In addition to definitive haemopoiesis, c-kit is required for commitment of ES cells to differentiate (Bashamboo *et al.*, 2006). Therefore c-kit expression in Flk-1 null ES cell colonies at days 5 and 10 in the haemopoietic plaque assay does not necessarily indicate differentiation of the haemopoietic lineage.

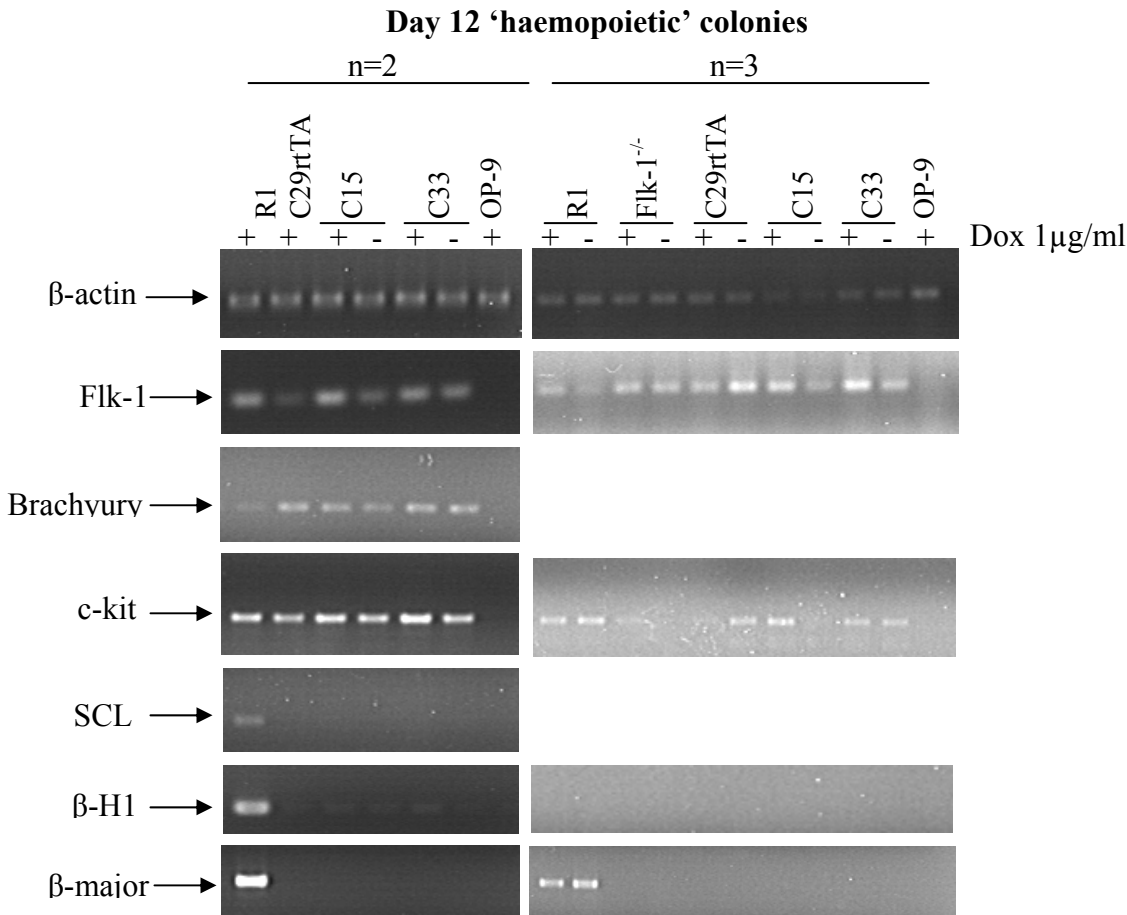
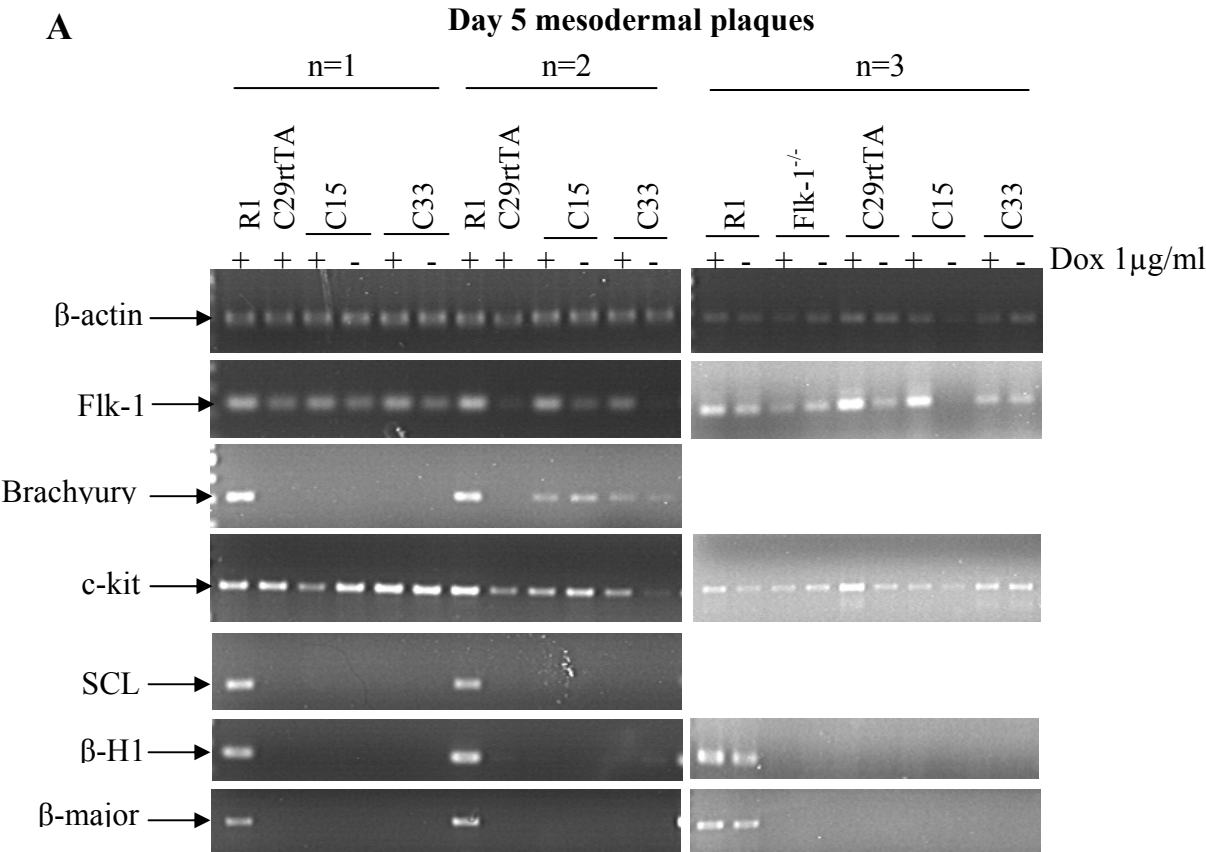


Figure 5-15 continued overleaf

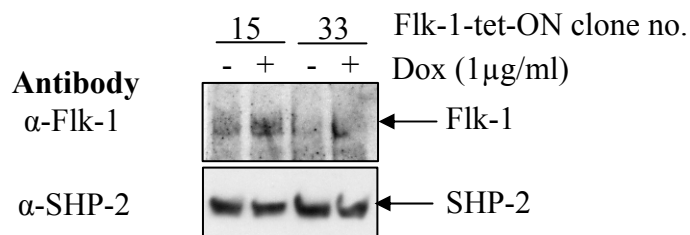
B

Figure 5-15 Expression of mesodermal and haemopoietic markers by wild type, Flk-1^{-/-}, c29rtTA and Flk-1-Tet-on ES cells differentiated on an OP9 stromal cell layer. ES cells were differentiated on an OP9 stromal cell layer for 5 days to form mesodermal plaques. Day 5 colonies were disaggregated and plated onto fresh OP9s to promote differentiation of haemopoietic colonies. Doxycycline, when added, was added at day 2.5 and re-added at day 5 when cells were re-plated. **A)** RNA was extracted from ES-derived colonies at day 5 and day 12 and from over-confluent OP9 cells to control for the presence of contaminating OP9 RNA. RNA was reverse transcribed to produce cDNA from which mesodermal and haemopoietic gene products were amplified using specific primers (see Table 2-9) to amplify mesodermal and haemopoietic gene products to determine cell fate. The expression of β -actin was used as a control as its expression remains unchanged regardless of treatment. Right and left hand panels from day 5 mesodermal plaques were run on the same gels as right and left hand panels showing marker expression in day 12 colonies. **B)** Differentiated Flk-1-Tet-on ES cells cultured in the presence and absence of doxycycline were lysed at day 12. Whole cell lysates were immunoblotted with an anti-Flk-1 antibody. Blots were stripped and re-probed with an anti-SHP-2 antibody to check for equal loading.

5.5 Discussion

Molecular cloning was used to produce ES cell lines which inducibly expressed the Flk-1 receptor. These cell lines were characterised and used to begin to investigate the importance and timing of Flk-1 signalling in cardiac and haemopoietic differentiation. Results are summarised below:

5.5.1 Summary

- A Baf/3 cell line constitutively expressing Flk-1 under the control of the CMV promoter was generated.
- Flk-1-Tet-on ES cell lines in which Flk-1 is expressed only in the presence of doxycycline were generated by stably transfecting Flk-1^{-/-} ES cells with both the reverse tetracycline-transactivator and the Flk-1 gene under the control of the tetracycline response element. Flk-1 expression exhibited doxycycline dose-dependence and was maintained for at least 4 days following induction in ES cells. VEGF stimulation of doxycycline treated Flk-1-Tet-on ES cells elicited auto-phosphorylation of tyrosine 951 over a time period identical to that seen in Flk-1 expressing embryoid-body derived cells.
- Flk-1 surface expression in differentiating Flk-1-Tet-on ES clones cultured in the presence of doxycycline was less robust and declined sharply between days 2 and 3 of differentiation.
- Flk-1^{-/-} ES cells stably transfected with the reverse tet-transactivator (clone 29) exhibited a marked inhibition in differentiation to form beating cardiomyocytes when incubated in the presence of doxycycline.
- Addition of doxycycline to Flk-1-Tet-on ES cells at day 2.5 of OP-9 co-culture failed to rescue the defect in haemopoietic development exhibited by Flk-1^{-/-} ES cells in this assay.

5.5.2 Inducible Flk-1 expression in Flk-1-tet-ON ES clones undergoing differentiation.

Flk-1 surface expression in Flk-1tet-ON ES cells was not maintained beyond 2 days in differentiating ES cells. Several differentiation assays were used to investigate duration of expression; embryoid bodies differentiated in either semi-solid or liquid medium and ES cells differentiated in monolayer by LIF removal. Flk-1 expression on day 3 was higher in both clones differentiated in monolayer by LIF removal but was still lower than on day 2 and was absent at day 4. Preliminary experiments in suspension embryoid bodies indicated that addition of dox at day 2 was unable to promote or maintain Flk-1 expression to day 4. Immunoblotting revealed that despite a lack of Flk-1 surface expression in day 4 embryoid bodies Flk-1 protein was still expressed. The decline in Flk-1 expression could be occurring for a number of reasons:

- **Degradation of doxycycline causing the doxycycline concentration available to fall below that required for maximal Flk-1 induction.**

The half-life of doxycycline is 24 hours. In the differentiation assays it was added at 1µg/ml. Maximal inducible Flk-1 expression was attained with between 0.1 and 0.5µg/ml doxycycline in Flk-1-tet-ON ES cells therefore doxycycline levels could fall below those needed to maintain expression by day 2. However, initial application of doxycycline in Flk-1-Tet-on ES cells maintained in a self-renewing state in the presence of LIF is sufficient to maintain expression for at least 4 days and preliminary experiments in which fresh dox was added after 2 days did not alleviate the decrease in expression suggesting that the degradation of dox in the system cannot fully explain the decline in Flk-1 surface expression after 2 days of differentiation.

- **Inability of doxycycline to penetrate embryoid bodies.**

As embryoid bodies increase in size it is possible that doxycycline is unable to penetrate to reach cells within the embryoid bodies. This may explain the difference between inducible Flk-1 expression in cells differentiated in monolayer cultures in the absence of LIF versus those differentiated in embryoid bodies. However, previous experience of the tet-system suggests that this is unlikely to pose a problem

as early as day 3 or 4 of embryoid body development. In previous work in which the Tet-off system was used to inducibly express SHP-1 no SHP-1 expression was seen in embryoid bodies as late as day 6 suggesting that tet was still able to reach cells within the embryoid bodies to repress SHP-1 expression (Paling and Welham, 2005). More over, inducible Flk-1 expression also declines in Flk-1-Tet-on ES cells differentiated by the removal of LIF, a system in which access of doxycycline to differentiating cells is unimpeded

- **Internalisation of Flk-1**

It is possible that overall cellular levels of Flk-1 protein do not decline but that instead Flk-1 is either internalised or fails to reach the cell membrane. Immunoblotting could be used to further investigate relative Flk-1 protein levels during differentiation to clarify whether there is a genuine decrease in total Flk-1 protein levels. Gampel *et al.*, in work performed using adult human endothelial cells, found that stimulation of cells with VEGF increased receptor cycling and thus increased Flk-1 expression at the surface (Gampel et al., 2006). Culture of embryoid bodies in the presence of both doxycycline and exogenous VEGF however, failed to increase Flk-1 surface expression in day 2 and day 3 suspension embryoid bodies over levels seen in EBs cultured in the presence of doxycycline alone.

- **Down-regulation of reverse-tet-transactivator expression upon differentiation.**

If the decrease in Flk-1 protein levels during differentiation is genuine it is likely due to the position of integration of either the reverse-tet-transactivator or the tet-response element within the genome. The decrease in Flk-1 expression upon differentiation is seen in two independent clones in which the response plasmid underwent two separate recombination events. Additionally, the tet-response plasmid is insulated with chicken β -globin chromatin insulators thus it is more likely be due to the site of integration of the regulator plasmid that drives expression of irtTA, which is the same in both clones. It is possible that the chromosomal integration site of the reverse-tet-transactivator in c29rtTA occurred in close proximity to genes or cis-acting elements that are down-regulated or repressed during differentiation, causing a decrease in expression of the reverse-transactivator itself. This could be

investigated using semi-quantitative RT-PCR or qPCR to monitor expression of the rtTA transcript during differentiation.

Further work is required to fully characterise the ability of Flk-1-Tet-on ES cells to maintain inducible Flk-1 expression during differentiation in a variety of systems. To confirm whether Flk-1 protein levels themselves decline during differentiation a time-course of Flk-1 protein expression in doxycycline induced Flk-1-Tet-on ES cells undergoing differentiation in both embryoid bodies and ES cells cultured in the absence of LIF would need to be performed. If, as seems likely, the decrease in Flk-1 surface expression correlates with a decrease in Flk-1 protein expression, which cannot be rescued by further addition of dox the cause of this down-regulation would need to be further investigated. It is possible that stable transfection of tettk-Flk-1-pA into a different rtTA Flk-1^{-/-} clone could create an inducible cell line which is able to maintain Flk-1 expression in both self-renewing and differentiating states. An alternative approach would be to use homologous recombination to integrate the regulator construct into the ROSA26 locus to enable maintenance of expression during differentiation (Masui et al., 2005; Zambrowicz et al., 1997).

5.5.3 Inability of c29rtTA to differentiate to the cardiomyocyte lineage in the presence of doxycycline

C29irtTA is a Flk-1^{-/-} ES cell line stably transfected with the reverse-tet-transactivator. It was used as the parental cell line from which the Flk-1-Tet-on ES cell lines expressing Flk-1 under control of the tet-response element were derived. During experiments characterising the ability of doxycycline-induced Flk-1 expression in Flk-1-Tet-on ES clones to rescue the delay in cardiomyocyte differentiation seen in Flk-1^{-/-} ES cells it was noted that addition of doxycycline caused a marked inhibition in the ability of the cells to form *any* beating cardiomyocytes. Further investigation revealed that doxycycline also inhibited cardiomyocyte formation in c29irtTA ES cells ruling out Flk-1 up-regulation as a cause of the phenotype. Previous studies in the lab have indicated that tetracycline itself can effect the expression of some genes involved in cardiac differentiation including Epb4.1 (Belinda Bateman, *pers. comm.*). However, addition of doxycycline

to R1 wild type ES cells or Flk-1^{-/-} ES cells caused no inhibition in cardiomyocyte formation and the addition of doxycycline to c29irtTA self-renewing ES cells did not appear to decrease proliferation or induce apoptosis. This suggests that the doxycycline-induced inhibition of cardiomyocyte differentiation is mediated by binding of doxycycline to the reverse-tet-transactivator and not the presence of dox itself. There is no tetracycline response element in this cell line therefore any effects mediated by the reverse-tet-transactivator would be off-target. irtTA is a fusion between the bacterial TetR and the viral VP16 transactivation domain. ES cells are very sensitive to toxicity of proteins with strong activation domains such as VP16 (Masui et al., 2005). VP16 could have affected cardiomyocyte differentiation by one of two mechanisms; transcriptional ‘squenching’ by the VP16 transactivation domain which occurs as a result of titration of components of the transcriptional machinery away from their respective intracellular pools interfering with transcription of non-target genes (Gilbert et al., 1993; Gill and Ptashne, 1988) or interaction of doxycycline-bound irtTA with non-target sequences allowing the transactivational domain of VP16 to repress or activate selective genes and abolishing the ability of c29irtTA ES cells to differentiate towards the cardiomyocyte lineage.

To further investigate this phenomenon the effect of doxycycline on the ability of other rtTA clones to differentiate to form cardiomyocytes could be investigated. This would help to elucidate whether the effect is mediated by the reverse-transactivator rather than doxycycline itself and clarify whether it is caused by the integration site of the reverse-tet-transactivator and is therefore unique to this clone. Whatever the mechanism of doxycycline-induced blockage of cardiomyocyte differentiation in c29irtTA ES cells the phenotype could enable study of genes important for cardiomyocyte differentiation. Initial characterisation of the point at which differentiation ceases could be carried out using semi-quantitative RT-PCR or q-PCR to investigate expression of markers of self-renewal (including Oct-4 and *Nanog*), mesodermal differentiation (*Brachyury*) and cardiomyocyte differentiation (*Nkx2.5*, *Gata4*, bFGF, cardiac troponin I and, *Myhc* (myosin heavy chain)). This could potentially be followed by chromatin-immunoprecipitation (ChIP) of genes bound to irtTA in cardiomyocyte cultures of c29irtTA ES cells cultured in the presence and absence of doxycycline using a polyclonal antibody which recognises the transactivation domain of VP16.

5.5.4 Ability of Flk-1-Tet-on ES cells to rescue the defect in haemopoietic differentiation of Flk-1^{-/-} ES cells in the haemopoietic plaque assay

Flk-1^{-/-} ES cells are unable to differentiate to form haemopoietic plaques in OP9 co-culture (Hidaka et al., 1999). The Flk-1-Tet-on ES cell lines were created to investigate the importance and timing of Flk-1 expression in systems such as this. Initially, 1µg/ml doxycycline was used to induce Flk-1 expression at day 3 of the assay to attempt rescue of haemopoietic differentiation. Analysis of expression of the haemopoietic markers *Scf*, β-H1-globin and β-major indicated that the rescue had been unsuccessful. Further investigation of Flk-1 protein expression in day 12 Flk-1-Tet-on/OP9 co-cultures indicated some expression of Flk-1 at this time point in the dox-treated samples, though it is possible that the levels of induced Flk-1 expression may not have been high enough to rescue haemopoietic differentiation. A comparison of dox-induced Flk-1 protein levels with Flk-1 protein levels in wild type R1 cultures on day 5 and 12 could help give a more accurate picture of the levels of dox-induced Flk-1 expression.

Another method of investigating the temporal requirement for Flk-1 signalling in haemopoietic differentiation in the haemopoietic plaque assay could be the use of small molecule Flk-1 inhibitors added at different time points. This approach is less flexible as it allows only for the inhibition of Flk-1 signalling and could not be used to investigate the effect of maintenance of signalling or early induction of signalling. This approach may be appropriate in investigating the temporal requirement of Flk-1 in haemopoietic differentiation as Flk-1 signalling is generally accepted to act early in the migration and expansion of haemopoietic progenitors (Schuh et al., 1999). However, it would not be as useful in investigation of the temporal requirement of Flk-1 expression in the differentiation of endothelial progenitors in which expression is maintained and is important for endothelial function (Zachary, 2001).

5.5.5 Conclusions

ES cell clones which upon addition of doxycycline inducibly express Flk-1 have been successfully created. Though initial characterisation of these lines was promising, surprisingly, their performance in differentiation assays was less so. This study has illustrated the importance of both robust characterisation of transfected cell lines and the inclusion of appropriate controls in such experiments. The main issues encountered in the use of the Flk-1-Tet-on ES cell lines created appear to relate to the integration of the reverse-tet-transactivator. Characterisation of cell lines expressing the (reverse)-tet-transactivator without a tet-response plasmid is difficult as no functional read out of tet-transactivator activity is available. A more straightforward way to circumvent potential issues with the site of integration of the (reverse)-tet-transactivator may be to transfect the tet-response plasmid onto more than one clone containing the transactivator to minimise the likelihood of positional effects or to generate an ES cell line in which rtTA expression is targeted to the ROSA26 locus, which is known to be expressed in a stable manner in most mouse tissues.

Though the issues encountered in the behaviour of the tet-system could not have been predicted at the outset of these studies, the cell lines created could still be used for short-term expression of Flk-1, for study of Flk-1 signalling at different stages of development and in the investigation of the effect of upregulating Flk-1 expression in self-renewing ES cells.

Chapter 6

General Discussion

6.1 The role of Flk-1 signalling in an embryoid-body model of development

Signalling mediated by the receptor tyrosine kinase KDR (human Flk-1) in adult somatic cells has garnered much research as KDR mediates VEGF-induced angiogenesis, important in a number of pathological conditions. These include cancer, in which the angiogenic contribution of KDR signalling to the growth of solid tumours is a major target in cancer therapy (Tonini et al., 2003), diabetic retinopathy, a complication of type I and type II diabetes and the underlying cause of which is deregulated angiogenesis (Frank, 2004). Genetic knockout of Flk-1 in mice results in embryonic lethality by day E9.5 due to a lack of formation of the vasculature and blood (Shalaby et al., 1995; Shalaby et al., 1997) demonstrating that Flk-1 signalling also plays a key role in embryonic development. However, despite extensive knowledge of signalling via KDR in adult somatic cells (Zachary, 2001), little is known about the signalling pathways activated by Flk-1 during development and the importance of these signalling pathways during embryogenesis. In this study signalling mediated downstream of Flk-1 in an embryoid body model of development was characterised and the functional importance of these signals in the differentiation of haemopoietic, endothelial and cardiomyocyte lineages was investigated. A summary of the findings is provided in Figure 6-1.

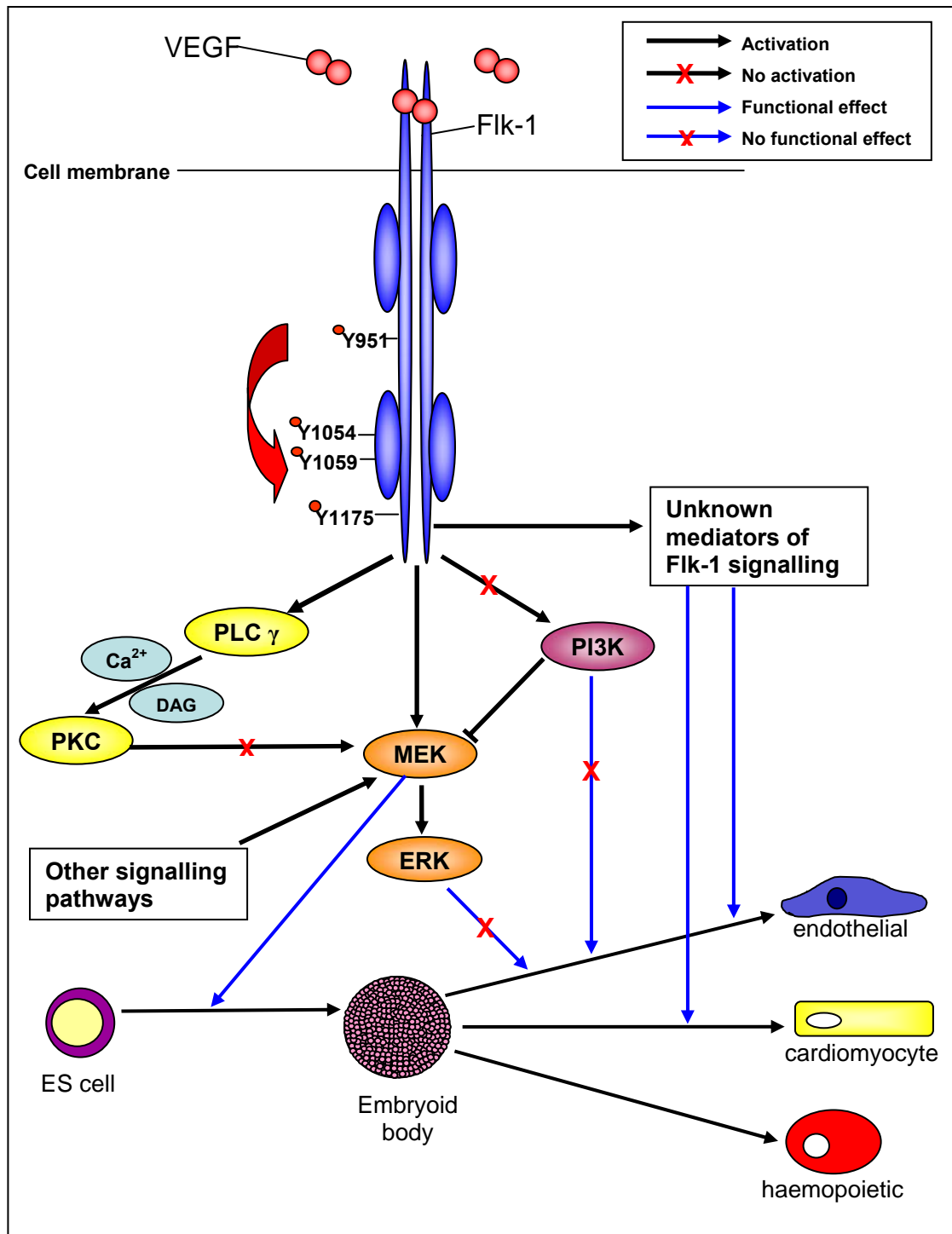


Figure 6-1 Model of Flk-1 signalling and its functional involvement in embryonic stem cell differentiation towards committed cells of the mesodermal lineage. Stimulation of murine Flk-1 in embryoid body derived-cells potentiates activation of PKC and MAPK signalling but does not appear to activate PI3K signalling. Flk-1 signalling is required for differentiation of the endothelial lineage but this requirement is not mediated through activation of the MAPK pathway or PI3K pathways. Flk-1 null ES cells exhibit delayed differentiation towards the cardiomyocyte lineage implying Flk-1 involvement in its differentiation.

Functional characterisation of Flk-1 in *in vitro* models of endothelial and cardiomyocyte differentiation was performed using Flk-1 null ES cells and the Flk-1 inhibitors, VEGFR2 kinase inhibitor-1 and VEGFR2 kinase inhibitor-2. An essential role for Flk-1 in vessel formation both in the adult (Shibuya and Claesson-Welsh, 2006) and the embryo (Shalaby et al., 1995) have been previously demonstrated. Results from the sprouting embryoid body assay also demonstrate involvement of Flk-1 in endothelial differentiation indicating that this assay provides a good model system in which to investigate the role of signalling downstream of Flk-1 during endothelial differentiation. Further dissection of the sprouting embryoid body assay utilising inhibition of Flk-1 with VEGFR2 kinase inhibitor -1 and VEGFR2 kinase inhibitor-2 during either the endothelial specification phase or the angiogenic expansion phase demonstrated individual requirements for Flk-1 in both endothelial specification and angiogenic expansion.

To investigate the signalling pathways activated downstream of Flk-1 populations of dissociated day 5 embryoid bodies, containing approximately 50% Flk-1-expressing cells, were stimulated with VEGF. Downstream signalling was characterised by immunoblotting of whole cell lysates. Transient activation of the MAPK and PLC γ signalling pathways was observed with phosphorylation levels of both PKC (classical and novel isoforms) and ERK1/2 peaking at 2 minutes VEGF stimulation and declining to basal levels by 10 minutes. This closely follows the pattern of VEGF-induced signalling seen in adult endothelial cells (Doanes et al., 1999; Takahashi and Shibuya, 1997; Yashima et al., 2001). In some adult endothelial cells activation of the MAPK pathway upon VEGF stimulation is thought to be mediated by PLC γ (Yashima et al., 2001). Inhibition of PKC with Calphostin C provoked no decrease in phosphorylation of ERK1/2, a downstream effector of MAPK signalling, suggesting that this is not the case in embryoid body-derived cells.

Further characterisation of MAPK signalling during differentiation was carried out using the MEK inhibitors PD98059 and U0126 in embryoid body-based assays. Complete inhibition of MEK-1 and 2 with 10 μ M U0126 abolished the ability of ES cells to differentiate to form embryoid bodies. Incubation of cells with 5 μ M U0126 or 50 μ M PD98059 led to decreased efficiency of embryoid body formation and a decrease in embryoid body size, suggesting a concentration dependent requirement

for MAPK signalling in commitment of ES cells to differentiation and in their subsequent proliferation. This data correlates well with experiments performed by Burdon *et al.*, which demonstrated increased ES cell renewal upon inhibition of the MAPK pathway with PD98059 (Burdon *et al.*, 1999). Though MAPK signalling is demonstrably important for differentiation it is unlikely that activation of the pathway early in embryoid body formation is mediated by Flk-1 as Flk-1 expression is not detected until day 3 of differentiation.

Partial inhibition of MAPK signalling using 5 μ M U0126 or 50 μ M PD98059 had no effect on endothelial sprouting from day 10 embryoid bodies suggesting that there is no requirement for MAPK signalling in the specification of endothelial cells or in their expansion. In genetic studies, MEK-2 knockout mice were viable (Belanger *et al.*, 2003) and although MEK-1 knockout mice were embryonic lethal at day 10.5 this was due to defective vascularisation in the labyrinth region of the placenta and no effect on embryonic vascularisation was noted (Giroux *et al.*, 1999). The lack of effect on embryonic vascularisation in both MEK-1 and MEK-2 knockout mice supports the findings from the sprouting embryoid body assay. However, as no studies have been performed using MEK-1/MEK-2 double knockout mice the possibility of functional redundancy cannot be ruled out. Flk-1^{Y1173F/Y1173F} embryos, which recapitulate the Flk-1^{-/-} phenotype demonstrate little or no reduction in ERK1/2 phosphorylation at E8.5 detected by immunocytochemistry when compared to wild type or Flk-1^{+/-} embryos (Sakurai *et al.*, 2005). This suggests that MAPK activation at this embryonic stage (during which endothelial specification and vasculogenesis are occurring) is largely mediated through other receptors and provides further support for the results observed in the *in vitro* sprouting embryoid body assay.

No evidence of activation of PI3K signalling upon VEGF stimulation of embryoid body-derived cells was observed despite the importance of PI3K signalling downstream of Flk-1 in adult endothelial cells (Fujio and Walsh, 1999; Fulton *et al.*, 1999). Activation of PI3K was also investigated in VEGF-stimulated secondary blast cells and D4T embryoid body-derived endothelial cells but again no activation was detected. Since activation of the PI3K pathway downstream of Flk-1 in adult endothelial cells this pathway is very important for VEGF-induced angiogenesis

(Shiojima and Walsh, 2002) the effect of inhibiting PI3K on endothelial sprouting was investigated. Inhibition of PI3K signalling with 5 μ M LY294002 had no effect on endothelial sprouting suggesting that PI3K is not required for vasculogenesis or angiogenesis in embryoid bodies. This supports the previous finding that PI3K signalling is not activated downstream of VEGF signalling in embryoid body-derived cells. Studies using knockout mice lacking both the p85 α and β regulatory subunits (Brachmann et al., 2005) or either the p110 β or p110 δ catalytic subunits (Bi et al., 2002; Clayton et al., 2002; Okkenhaug et al., 2002) displayed no defect in vessel formation, although knock-out of p110 β was embryonic lethal before implantation masking any potential involvement in vasculogenesis. However, embryonic lethality at day 10.5 in the p110 α knockout mouse *is* caused by defects in vascularisation including “dilated vessels in the head, reduced branching morphogenesis in the endocardium, lack of hierarchical order of large and small branches in the yolk sac and impaired development of anterior cardinal veins” (Lelievre et al., 2005). These defects reportedly mimic several aspects of the *Tie2*^{-/-} phenotype and deficiency in the p110 α catalytic subunit also causes diminished *Tie2* expression causing the authors to postulate that PI3K functions as an upstream regulator of *Tie2* expression during mouse development (Lelievre et al., 2005). As the observed defects in vascularisation occur after the initiation of vasculogenesis this result supports my *in vitro* findings and points to a role for class IA PI3K in angiogenesis later in development which continues in the adult. The surprising finding that Flk-1 does not appear to activate PI3K signalling during early embryoid body development nor is PI3K involved in early endothelial differentiation and expansion suggests that Flk-1 signalling in the adult and the embryo may not be as similar as is often assumed (Hong et al., 2006).

Recent research has pointed to involvement of Flk-1 in cardiac differentiation (Boheler et al., 2002; Iida et al., 2005; Sachinidis et al., 2003b). Cardiogenic mesoderm is postulated to differentiate from a population within the embryoid body which lacks Flk-1 expression at day 3.25 but upregulates expression of Flk-1 by day 4.25 (Kattman et al., 2006; Kouskoff et al., 2005). However, despite a strong association between Flk-1 expression and cardiac differentiation a functional requirement for Flk-1 in cardiogenesis had not been proven. A comparison of cardiomyocyte differentiation in R1 parental and Flk-1 null ES cells revealed a delay

in formation of beating cardiomyocyte foci in Flk-1 null ES cells when compared to R1 parental ES cells. This indicates that Flk-1 promotes, but is not required for, cardiomyocyte differentiation suggesting that any absolute requirement for Flk-1 expression occurs at a different stage of cardiogenesis.

In order to further investigate the role of Flk-1 in haemopoiesis, endothelial differentiation and cardiomyocyte differentiation an inducible Flk-1-Tet-on ES cell line was created on a Flk-1^{-/-} parental background. This approach was designed to allow investigation of the temporal importance of Flk-1 signalling during differentiation. Additionally, mutations of tyrosines within the cytoplasmic tail of Flk-1 would have allowed characterisation of the phosphorylation sites required for activation of different signalling pathways and their importance during differentiation. However, a number of unexpected obstacles were encountered with this approach that could not have been predicted at the outset. First, surface expression of doxycycline-induced Flk-1 could not be sustained long-term upon induction of differentiation, significantly limiting the window during which analyses could be performed. Secondly, and very surprisingly, addition of doxycycline to Flk-1^{-/-} ES cells expressing TetR abolished cardiomyocyte differentiation. Addition of doxycycline had no effect on the ability of R1 wild type or Flk-1 null ES cells to form beating cardiomyocytes which implied that the dox-effect was mediated by rtTA. This made study of the temporal importance of Flk-1 during differentiation difficult but use of a similar approach could still yield valuable results (see section 6.2.4 for further discussion).

6.2 Future directions

During the course of this study several areas that warrant further research have arisen. These range from further characterisation of Flk-1 signalling in embryoid body-derived cells to new genetic approaches to study the temporal effects of Flk-1 expression in models of early development.

6.2.1 Flk-1-mediated signalling in *in vitro* models of development

Investigation of Flk-1-mediated signalling in embryoid body-derived cells revealed activation of the MAPK and PLC signalling pathways upon VEGF stimulation and indicated that MAPK activation was not mediated through PKC. To confirm non-involvement of PLC γ in VEGF-mediated MAPK activation further studies could be conducted using the PLC inhibitor U73122. Activation of Ras by VEGF could be investigated using Raf-1 pull-down followed by immunoblotting for Ras protein. siRNA or dominant negative Ras (Yamamoto et al., 1999) could then be used to investigate the Ras/Raf dependency of VEGF-induced MAPK signalling. Further characterisation of VEGF-stimulated signalling in embryoid body-derived cells, including characterisation of potential activation of p38 MAPK and the involvement of adapter proteins such as Shb (Holmqvist et al., 2004; Rolny et al., 2005) and VRAP (Wu et al., 2000b) in coupling Flk-1 to downstream signalling pathways, could be performed using immunoprecipitation and siRNA-based approaches. Further genetic approaches for the study of Flk-1-mediated signalling are discussed in 6.2.4.

6.2.2 Signalling pathways involved in Flk-1 mediated commitment and expansion of endothelial cells

As neither PI3K nor MAPK signalling mediate the involvement of Flk-1 in endothelial differentiation the identity of the signalling pathway(s) involved is still of interest. Other downstream effectors of Flk-1 signalling in adult endothelial cells which may mediate endothelial differentiation and vasculogenesis include p38

MAPK (Lamallice et al., 2006; Yashima et al., 2001) and PLC γ 1 (Takahashi et al., 2001; Takahashi and Shibuya, 1997). The p38 MAPK pathway has been implicated in endothelial migration in adult angiogenesis (Yashima et al., 2001), but use of the pharmacological inhibitor of p38 MAPK, SB202190, enhanced neovascularisation in chick chorioallantoic membrane (Matsumoto et al., 2002). However, the PLC γ 1 knockout mouse is phenotypically similar to the Flk-1^{-/-} mouse, dying at E10 (0.5-1 day later than the Flk-1^{-/-} mouse) due to vascular and erythroid defects (Liao et al., 2001). This pathway therefore seems a good target for further investigation. Initial characterisation of PLC γ 1 null ES cells (Shirane et al., 2001) in the embryoid body-sprouting assay would validate involvement of PLC γ 1 in endothelial differentiation and expansion. Inhibition of PLC with an inhibitor such as U73122 (Bleasdale et al., 1989) could then be used to provide further information concerning whether any PLC-mediated effect occurred during specification of endothelial cells or their expansion.

6.2.3 Characterisation of the Flk-1-mediated delay in cardiomyocyte differentiation

Flk-1 null ES cells are delayed in their ability to form beating foci of cardiomyocytes when compared with R1 wild type ES cells. Further characterisation of the role of Flk-1 in cardiomyocyte formation could initially involve use of Flk-1 inhibitors to validate the results gained from use of Flk-1 null ES cells and investigate when inhibition of Flk-1 is required to cause the delay in cardiomyocyte differentiation. The delay in initiation of beating in the Flk-1 null cardiomyocyte cultures may be caused by deficiency in activation of a signalling pathway(s) downstream of Flk-1, which can be overcome by upregulation of signalling through a related receptor, such as FGFR1 (Mima et al., 1995). Possible candidate signalling molecules which may be responsible for the delay in cardiomyocyte differentiation include; PLC γ 1 (Rottbauer et al., 2005), eNOS (Kanno et al., 2004) or p38 MAPK (Aouadi et al., 2006). Use of inhibitors of these signalling pathways (for example with U73122, L-NAME and PD169316 respectively) could enable validation of any involvement in the Flk-1 null phenotype. Further characterisation could involve including comparative analysis of expression of genes including *Nkx2.5*, *Fgfr1*, *Gata4*, bFGF,

cardiac troponin I, *Myhc* (myosin heavy chain) and eNOS between Flk-1 null and R1 ES cells during cardiomyocyte differentiation. This would allow investigation of any association between the delay in cardiomyocyte beating and a delay in upregulation of cardiomyocyte-related genes and would test whether the delay is in fact abrogated by increased bFGF or FGFR1 expression.

6.2.4 Use of genetic approaches to study the roles of Flk-1 signalling in haemopoietic, endothelial and cardiomyocyte differentiation

A system in which Flk-1 expression could be regulated would allow more detailed investigation of the temporal requirements of Flk-1 in haemopoietic, endothelial and cardiomyocyte differentiation. In addition, site-directed mutagenesis of tyrosines within the cytoplasmic domain of the Flk-1 receptor could enable investigation of the signalling pathways activated by phosphorylation of specific tyrosine residues using VEGF stimulation of embryoid body-derived cells. Following characterisation of signalling, the relative importance of specific tyrosine residues, and the signalling pathways they activate, could be assessed in functional assays evaluating haemopoietic, endothelial and cardiomyocyte differentiation. Unusually for a receptor tyrosine kinase much of the role of Flk-1 appears to be mediated by tyrosine 1173. The Flk-1^{Y1173F/Y1173F} mouse exhibits an almost identical phenotype to the Flk-1^{-/-} mouse (Sakurai et al., 2005) and phosphorylation of the corresponding tyrosine residue of KDR in adult endothelial cells has variously been reported to activate the PI3K pathway (Dayanir et al., 2001; Holmqvist et al., 2004) PLC signalling and MAPK signalling (Sakurai et al., 2005; Takahashi et al., 2001). During the course of this study I generated a Flk-1 construct in which tyrosine 1175 was mutated to phenylalanine. This mutated Flk-1 could be expressed constitutively or in an optimised Tet-regulatable expression system in Flk-1 null ES cells to investigate whether VEGF is still able to activate previously characterised Flk-1-mediated signalling pathways, including MAPK and PLC signalling. Other tyrosine residues of interest include tyrosine 1212, reported to be required for MAPK and PLC signalling (in contrast to findings by Takahashi *et al.*) (Meyer et al., 2002; Takahashi et al., 2001) and tyrosine 951, which reportedly couples to the adaptor protein Shb which

mediates activation of PI3K and endothelial migration in adult endothelial cells (Holmqvist et al., 2004).

Random integration of the reverse-tet-transactivator in the clone used for creation of the Flk-1 Tet-on ES cell line may have been responsible for the lack of sustained expression of doxycycline-induced Flk-1 upon differentiation. To prevent this, future studies could be conducted by targeting the tet-transactivator to the ROSA26 locus using homologous recombination (Masui et al., 2005; Zambrowicz et al., 1997). An alternative approach, utilising ES cells in which Flk-1 expression can be inducibly knocked down using an inducible shRNA system (Gupta et al., 2004; Hiraoka-Kanie et al., 2006) would allow investigation of the temporal importance of Flk-1 expression but could not be used to investigate the signalling pathways activated through different tyrosine residues in the receptor. Use of a recently described dual knockdown-rescue approach (Ivanova et al., 2006) would allow rescue of Flk-1 knockdown by Flk-1 with mutated tyrosine residues, thus facilitating investigation of the signalling mediated by, and functional importance of tyrosine residues within the cytoplasmic tail of Flk-1.

These studies have provide new insight into how signalling mediated by Flk-1 is coupled to functional responses in models of embryonic development and raised further questions that could be addressed in future studies.

Chapter 7

Literature

sources

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Chapter 8

Appendix – plasmid maps

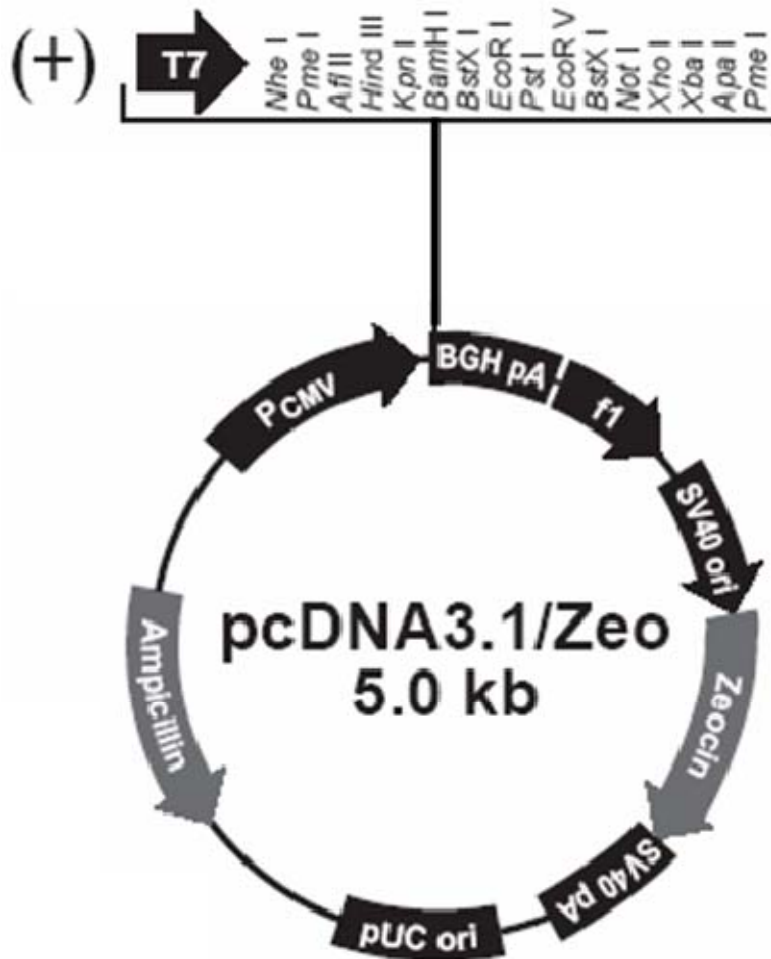
Descriptions of plasmids used in this study are given in Table 2-9 which is reproduced below. This Appendix contains additional plasmid information and plasmid maps

Table 2-9 Plasmids used in this study

Plasmid	Derivation	Use
pcDNA3.1Zeo (Invitrogen)	Mammalian expression plasmid containing the CMV early immediate promoter and Ampicillin and Zeocin resistance genes.	Tractable vector used for initial cloning of Flk-1.
pcDNA3.1Zeo-MS Flk-1	The Murine Flk-1 sequence ligated into pcDNA3.1zeo using the XbaI restriction site	Used to check viability of cloned Flk-1 sequence in BaF/3 cell line and for sequencing.
pCAGGS-irtTA- VP16-IRES-puro (Anastassiadis et al., 2002)	Reverse Tetracycline transactivator with puromycin resistance gene.	Enables regulated expression by addition of doxycycline of genes expressed in the tet response plasmid.
pKS-TetTK-pA (Anastassiadis et al., 2002)	Bluescript vector modified to encode tet operators, a thymidine kinase (TK) promoter and polyA tail.	Used for insertion of Flk-1 sequence into TetTK-pA.
pKS-TetTK-flk-1- pA	Flk-1 ligated into pKS-TetTK-pA using the SpeI restriction site	Used to insert TetTK-Flk-1-pA sequence into Inshygro vector.
pKS-Δp85-TetTK- pA	Myc tagged p85 subunit lacking the binding site for the catalytically active p110 component of PI3K ligated into pKS-TetTK-pA.	To determine the presence of functional tetracycline transactivator in stably transfected R1 ES cells using transient transfection.
pAcΔPHAkt-GFP- N1	pACGFP-N1 vector (Clontech) expressing GFP tagged Akt PH domain non-lipid binding mutant	Optimising transient transfection of R1 ES cells using flow cytometry to detect GFP expression
pInshygro (Anastassiadis et al., 2002)	Expression vector containing 4 chicken β-globin chromatin insulators flanking the multiple cloning site and a hygromycin resistance gene.	Insulators thought to block inappropriate action of regulatory elements reducing likelihood of mosaic expression. Hygromycin resistance enables selection.
pInshygro-TetTK- Flk-1-pA	TetTKFlk-1-pA blunt end ligated into the multiple cloning site of the SnaBI digested Inshygro plasmid	Construct linearised and transfected into Flk-1 ^{-/-} R1 ES cells stably expressing CAGGS-irtTA-VP16-IRES-puro

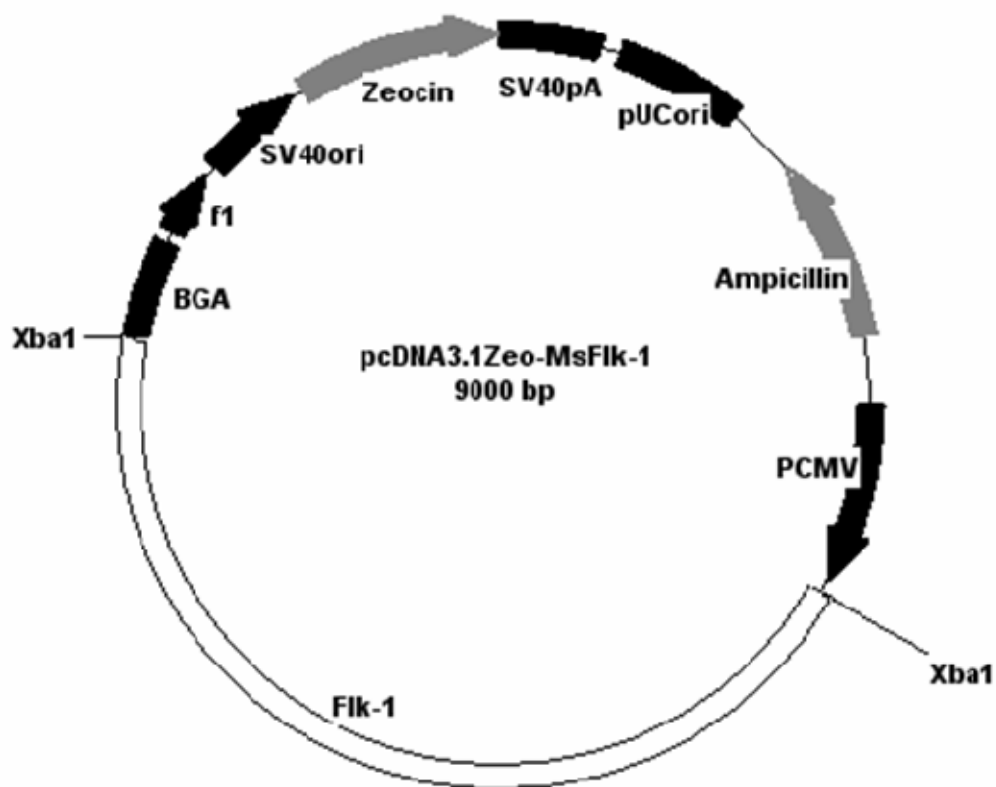
8.1 pcDNA3.1Zeo

Plasmid obtained from Invitrogen.

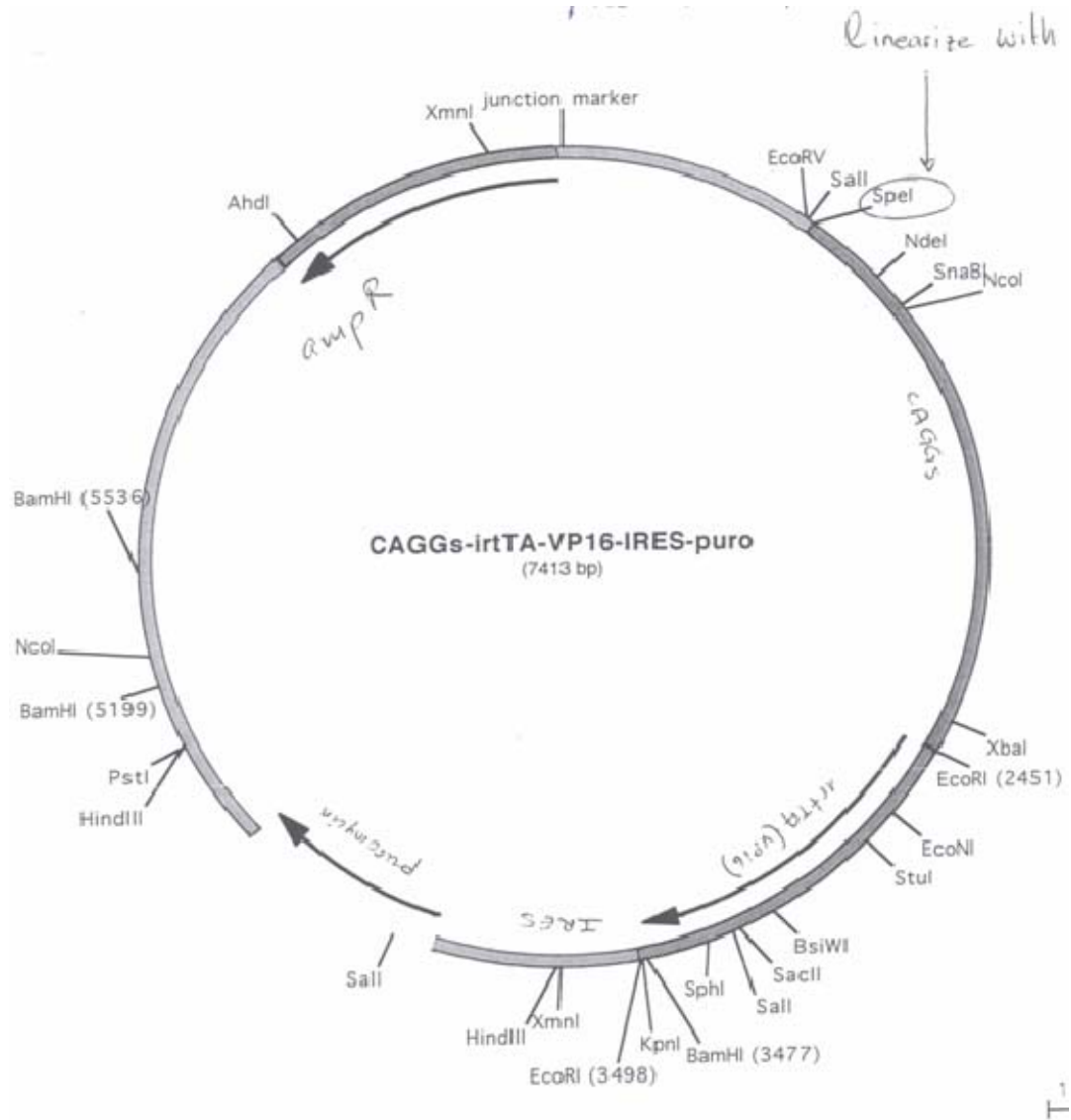


8.1.1 pcDNA3.1Zeo-Flk-1

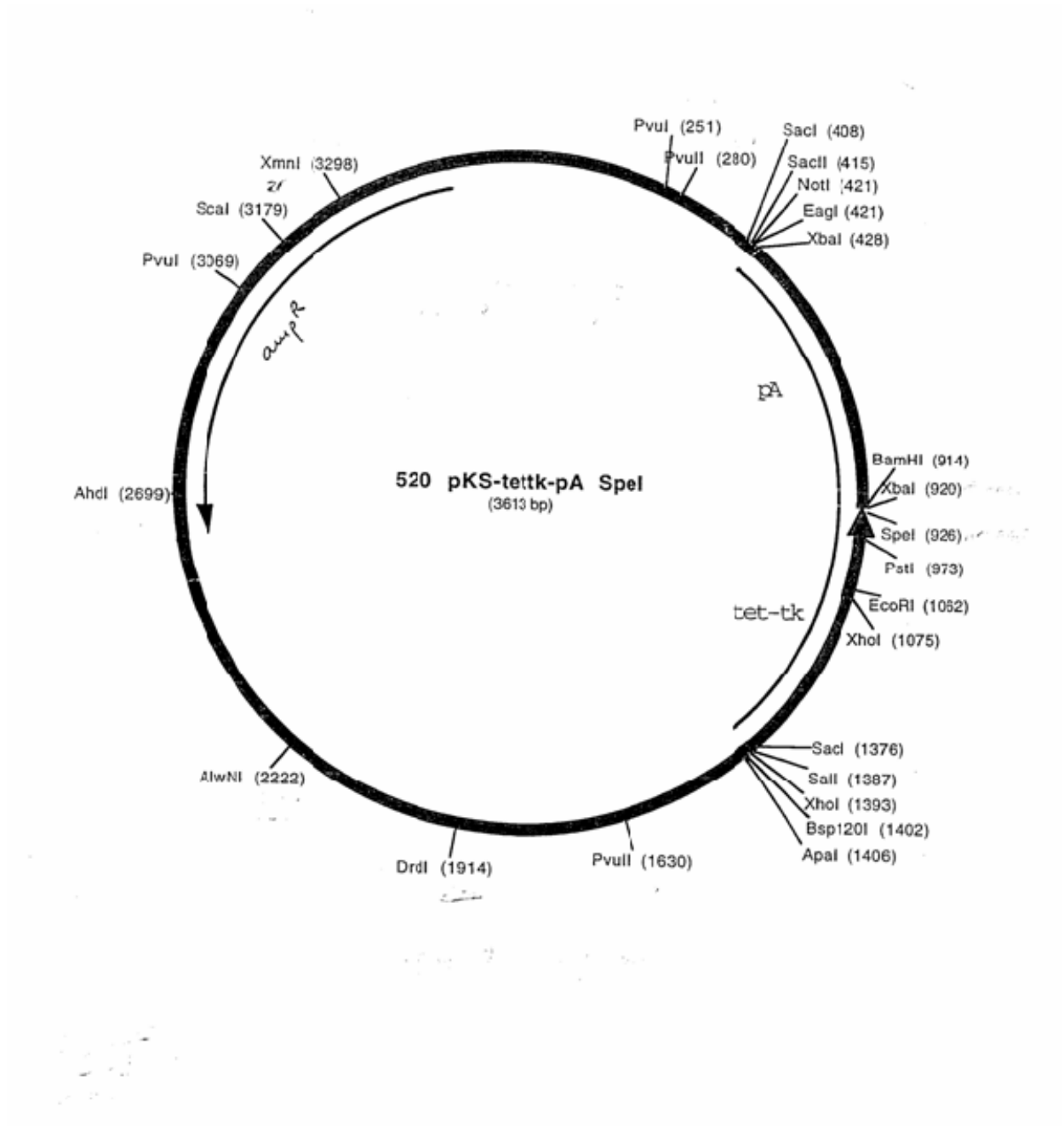
The pcDNA3.1Zeo plasmid was linearised with XbaI a non-cutter in the Flk-1 sequence. Flk-1 cDNA was amplified with primers designed to introduce 5' and 3' XbaI restriction sites. Following XbaI digestion the Flk-1 sequence was ligated into pcDNA3.1Zeo creating the plasmid illustrated below.



8.2 CAGGs-irtTA-VP16-IRES-puro

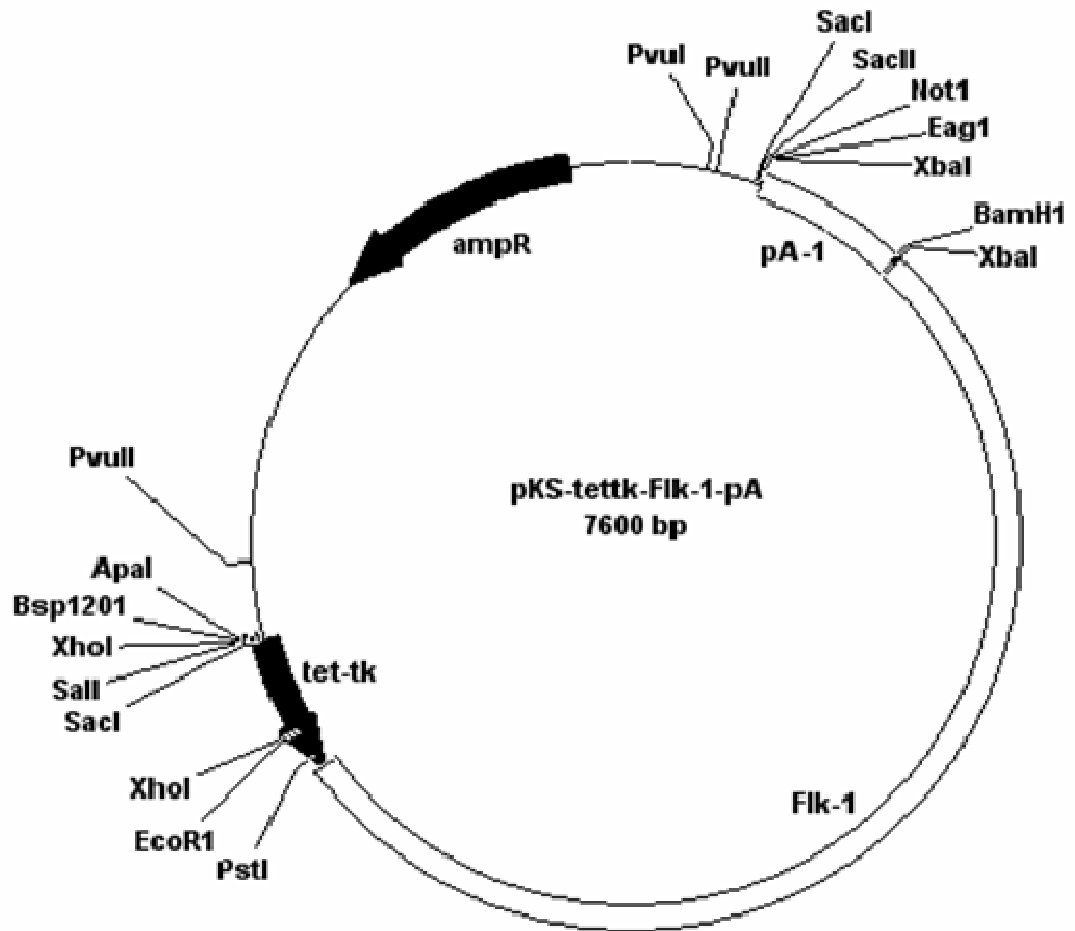


8.3 pKS-tettk-pA

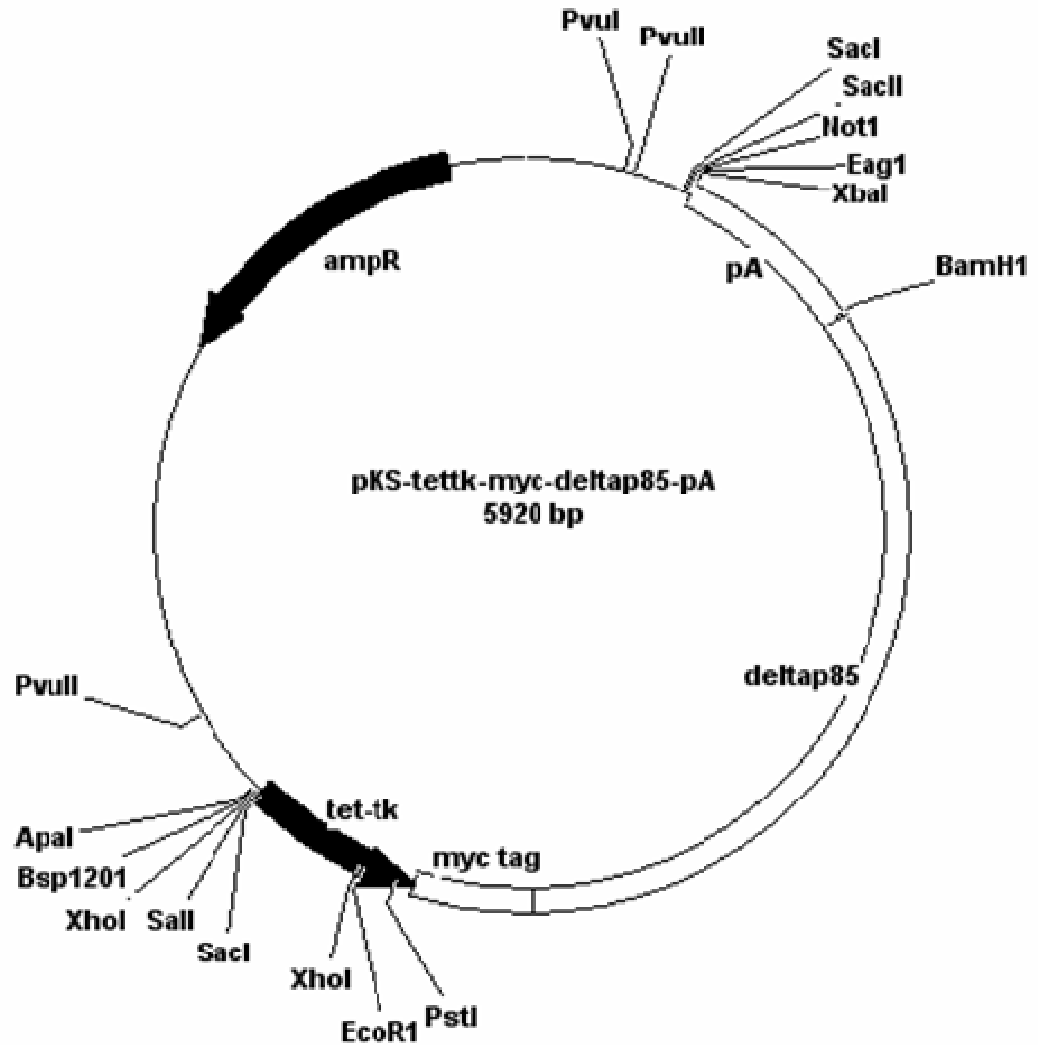


8.3.1 pKS-tettk-Flk-1-pA

Flk-1 was digested out of the pcDNA3.1Zeo-Flk-1 plasmid using Xba1. The pKS-tettk-pA plasmid was linearised using Spe1 creating compatible ‘sticky ends’ for ligation of Xba1 digested Flk-1 to create the following plasmid.



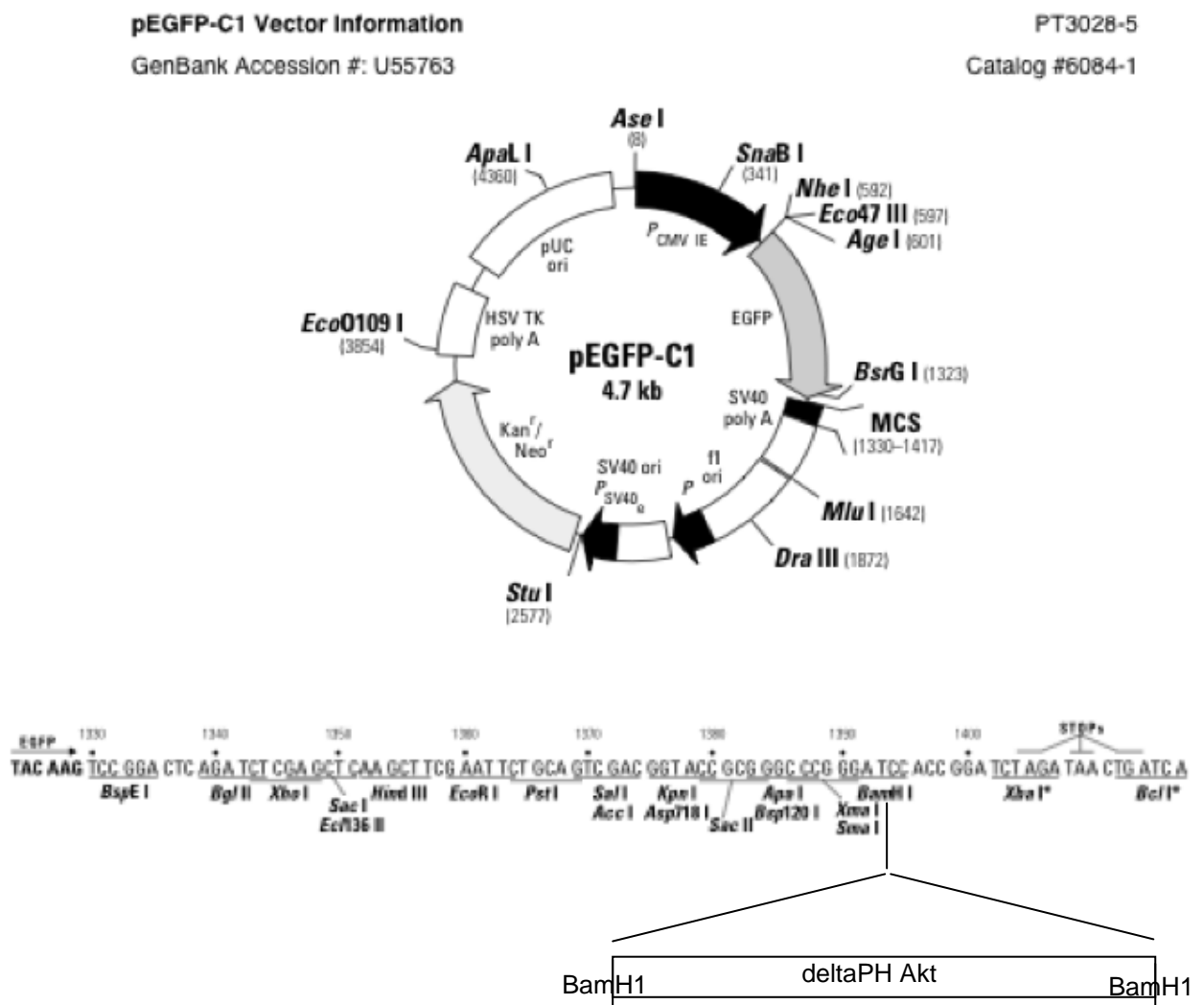
8.3.2 pKS-tettk-myc-deltap85-pA



8.4 pAcΔPHAkt-GFP-N1

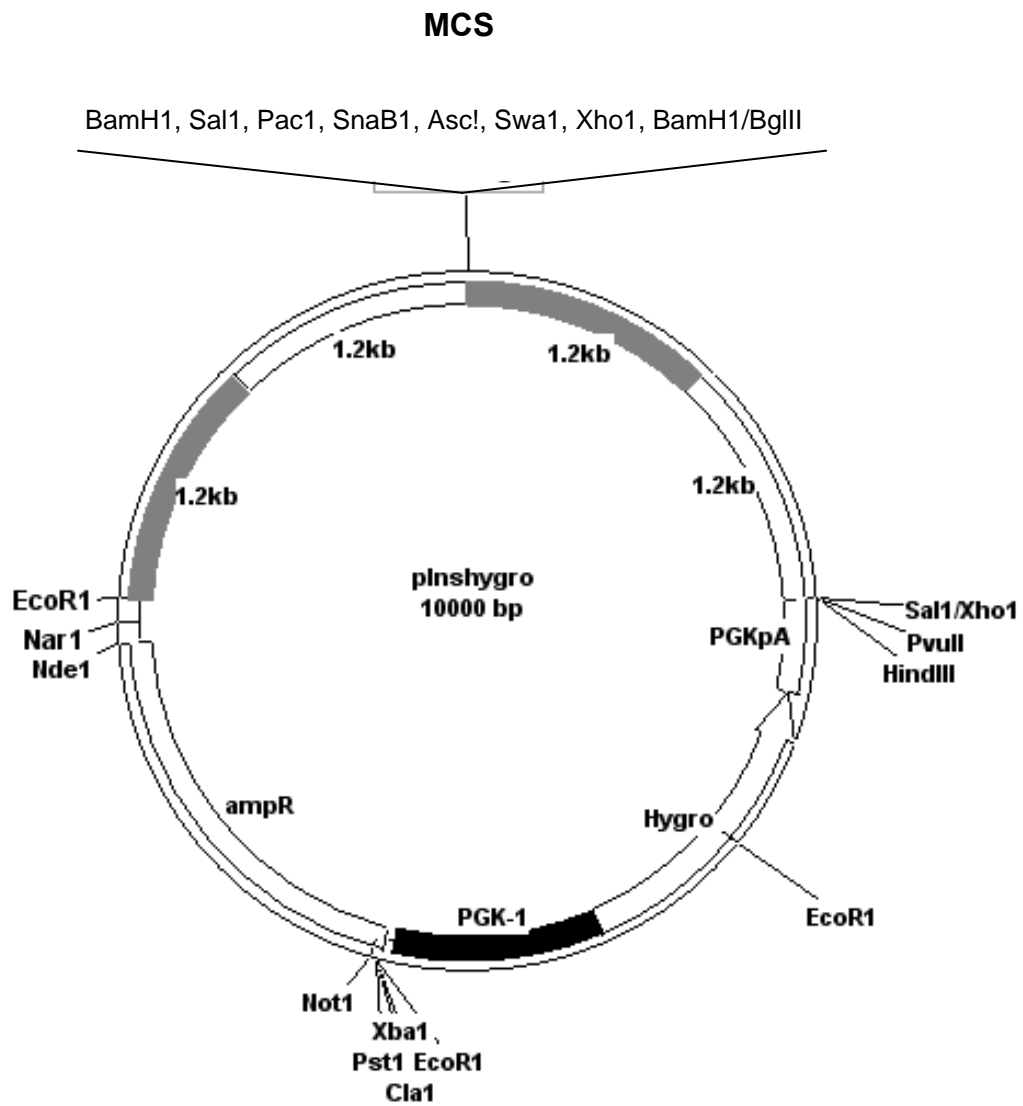
In this study GFP expression by this plasmid was exploited to allow for efficient screening of transiently transfected cells using flow cytometry in order to optimise transient transfection conditions in R1 ES cells.

The plasmid does however also contain the PH domain of Akt mutated to prevent lipid binding. This construct was flanked with BamH1 sites and ligated into the pEGFP-C1 plasmid following digestion with BamH1 which cuts in the multiple cloning site (MCS).



8.5 pInshygro

The 1.2kb elements are chicken β -globin chromatin insulators.



8.5.1 pInshygro-tettk-Flk-1-pA

To create the pInshygro-tettk-Flk-1-pA plasmid tettk-Flk-1-pA was digested out of the pKS-tettk-Flk-1-pA plasmid using PvuII. The tettk-Flk-1-pA was blunt end ligated into SnaBI linearised pInshygro between the flanking insulators creating the plasmid illustrated below.

